EFFECTS OF VITAMIN E AND HINDLIMB UNLOADING ON BONE COMPOSITION IN AGED RATS

By

TRACY LYNN RIGGS

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ON BONE COMPOSITION

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Thesis Approved:

Barbara J. Stoecker
Thesis Adviser

Elizabeth A Dorse

Andrea B. Arquitt

Donna J. Smith

Dean of the Graduate College

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RESEARCH PROBLEM

Introduction to the Problem

The World Health Organization defines osteoporosis as "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequential increase in fracture risk" (WHO Study Group 1994). Osteopenia, the loss of bone mass, is the result of altered bone remodelling. Bone remodelling is controlled through the activity of osteoblasts and osteoclasts. Osteoblasts are mononucleated cells derived from osteoprogenitor (stem) cells that synthesize and lay down new bone matrix. Osteoclasts are multinucleated cells of monocyte lineage that resorb old bone by secreting lysosomal enzymes, hydrogen peroxide, and free radicals into a resorptive compartment next to bone (Marks & Popoff 1988, Christenson 1997). Osteoclastic bone resorption paired with osteoblastic bone formation is part of the normal process of bone remodelling regulated by interactions between systemic and local factors (Mundy 1999).

As aging occurs, the rate of bone resorption tends to exceed the rate of formation, resulting in a net loss of bone. Bone mass rapidly increases during puberty and until 25 to 30 years of age, at which time peak bone mass is achieved (Parfitt et al. 1983). However, the progressive loss of bone generally begins at about age 35 in most humans (Baron 1999). Factors affecting bone resorption include an individual's genetic potential,

environmental influences, nutrition, and weight-bearing activity (Seifert & Watkins 1997).

In 1892, Julius Wolff proposed the idea that bone increased its density and strength in areas exposed to stress, while areas not physically stimulated become weaker and less dense (Gooch & Tennant 1997). Lack of physical stimulation, or skeletal unloading occurs primarily in circumstances of immobilization (such as paralysis), prolonged bedrest, or space flight.

Oxidative damage is another factor influencing bone resorption. Osteoclasts are generated from cells of the monocyte-macrophage lineage, which secrete oxygen-derived free radicals that are involved in the cellular formation and activation of the osteoclast itself (Garrett et al., 1990).

Other local factors responsible for influencing bone turnover are cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and prostaglandins of the E-2 series such as PGE_2 (Kenny & Prestwood 2000).

Nutrition is also very important in bone remodelling. In the past, research has focused primarily on the impact of vitamin D and minerals, such as calcium and phosphorus, on the skeleton. However, antioxidants have recently been suggested as playing a role in bone metabolism (Seifert & Watkins 1997).

Vitamin E is a fat-soluble antioxidant that functions as a free radical scavenger to prevent lipid peroxidation (Cohen & Meyer 1993). Vitamin E may suppress the production of cytokines and prostaglandins that are involved in osteoclastic bone resorption (Wu et al. 2001, van Tits et al. 2000, Devaraj & Jialal 1996, 2000). Vitamin E and other antioxidants may have a protective effect on bone (Xu et al.1995, Maiorano et al. 1999). However, minimal research has focused on vitamin E and its effect on bone metabolism in a model undergoing rapid bone loss.

Significance of Problem

The disability, mortality, and cost of osteoporosis represents a challenging public health problem. It is estimated that approximately one-half of all women and one-quarter of all men over age 50 are affected at an annual cost of over 10 billion dollars in the United States alone. Additionally, the direct healthcare costs of osteoporosis are estimated to be near 38 million dollars per day (Kenny & Prestwood, 2000). Due to the increasing median age, the diagnosis, treatment and monitoring of osteoporosis and other skeletal diseases has become a leading healthcare issue (Christenson 1997).

With prolonged stays in space becoming more common, bone loss resulting from exposure to microgravity is also an important biomedical concern. Microgravity induced osteoporosis was noted in the first human space flights in the early 70's. During the 237-day Soviet Soyuz T-10 mission, the three crewmembers lost an average of 13-19% of bone (Stupakov et al. 1984). Subsequently, Collett et al. (1997) found a 25% decrease in bone mineral density of two astronauts after a 6-month spaceflight. The microgravity induced bone loss in space is very similar to disuse-osteoporosis on earth and bone-remodeling markers have been extrapolated to either condition (Christenson 1997).

On earth, the hindlimb unloaded (HLU) animal model has proven useful to study the effects of near weightlessness on bone and other physiological parameters (Morey-Holton 1998). Previous studies of weightlessness on bone have focused primarily on parameters of bone metabolism without the addition of nutritional variables (Machwate et al. 1993, Keila et al. 1994, Moos et al. 1994, Wronski & Morey 1983). This experiment was designed to investigate the effects of vitamin E on bone metabolism in the hindlimb unloaded rat.

Objectives

The following objectives were developed to investigate the effects of skeletal unloading and three concentrations of vitamin E on bone in aged rats.

1. To determine if hindlimb unloading and vitamin E have any effect on bone mineral density or biochemical markers of bone remodelling in aged rats.
2. To investigate the changes in concentration of selected macro and trace elements in the bone of hindlimb unloaded vitamin E supplemented aged rats.
3. To explore the impact of hindlimb suspension and vitamin E status on marrow-derived bone cells.

Hypotheses

The following hypotheses were developed for this study.

1. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter the bone mineral area, bone mineral concentration, or bone mineral density in aged rats.
2. There will be no statistically significant effects of hindlimb unloading, vitamin E status, or their interaction on biochemical markers of bone remodelling (i.e. serum alkaline phosphatase, bone-specific alkaline phosphatase, serum osteocalcin, and serum tartrate-resistant acid phosphatase) in aged rats.
3. There will be no statistically significant difference in the total bone content or concentration of calcium, magnesium, copper, iron, and zinc due to hindlimb unloading, vitamin E status, or their interaction in aged rats.
4. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter the initial viability, alkaline phosphatase secretion, osteocalcin secretion, or tartrate-resistant acid phosphatase activity in marrow-derived bone cells from aged rats.

Limitations

During hindlimb unloading, the age of the animal is very important. Most of the research using the hindlimb unloaded rat has been performed on young growing rats. Hindlimb unloading in young rats results in impaired bone formation, with little or no

increase in bone resorption. In aged rats, skeletal unloading appears to increase bone resorption. However, the etiology of the increased resorption is not well defined. Although the data from young growing rats has provided valuable information regarding the effects of loading on bone, caution must be used when extrapolating the information to the adult human skeleton.

Format of Thesis

The experiment included in this thesis is organized as an individual manuscript and written using the Guide to Authors from the Journal of Nutrition (2000).

CHAPTER II

REVIEW OF LITERATURE

Bone

Bone is a connective tissue composed of specialized cells and an extracellular matrix. The extracellular matrix is formed by collagen fibers and noncollagenous proteins (Baron 1999). The homeostasis and development of bone is maintained by a delicate balance between bone formation and resorption (Lazner et al.1999). This equilibrium is controlled by the activities of osteoblasts and osteoclasts. Osteoblasts are cells responsible for building new bone by synthesizing collagen and protein for the matrix and promoting calcification (Marks & Popoff 1988). Osteoclasts are bone-lining cells that resorb old bone by producing and releasing lysosomal enzymes, hydrogen protons, and free radicals into a resorptive compartment next to bone, which dissolves the mineral and degrades the bone matrix. (Baron 1999, Marks & Popoff 1988).

In ideal physiologic circumstances, resorbed bone is immediately replaced with new bone by osteoblasts. However, there are many conditions in which bone resorption exceeds the rate of formation, leading to demineralization and an increased risk of fractures.

Age-associated osteopenia is one of the most common instances in which bone resorption exceeds the rate of bone formation. Although there are many factors involved,

a major cause appears to be a decrease in mechanical loading and usage (Bagi et al., 1993).

Local Regulators of Bone Metabolism

Prostaglandins are important local regulators in bone. They are formed from arachadonic acid via the cyclooxygenase pathway. Prostaglandin E_2 (PGE_2), is a known stimulator of bone resorption. Numerous studies have indicated that in the presence of PGE_2, osteoclasts are activated and resorption occurs (Collins and Chambers 1991, Kaji et al. 1996, Akatsu et al. 1989). However, the exact mechanism is still unclear.

Akatsu et al. (1989) hypothesized that certain prostaglandins stimulate bone resorption by promoting the recruitment of osteoclasts. They cultured bone marrow cells obtained from 7-9 week old male mice in varying concentrations of PGE_1, PGE_2, and $PGF_{2\alpha}$. Tartrate-resistant acid phosphatase staining was used to determine the development of osteoclastic cells and cyclic adenosine 3',5'-monophosphate (cAMP) concentration was also measured in the cultures. Results indicated that PGE_1 and PGE_2 were dose-dependently the most potent in inducing the formation of osteoclast-like cells. They also determined the order of the potency of prostaglandins in inducing bone resorption correlated with increasing cAMP production by bone cells, suggesting that prostaglandins induce osteoclast-like cell formation by a mechansim involving cAMP. These findings were similar to those of Kaji et al. (1996) and Yamaguchi et al. (1998), that, in culture, PGE_2 stimulates osteoclast-like cell formation through an increase in cAMP levels.

Cytokines are molecules released by macrophages or lymphocytes which modulate various cellular activities and play an important role in the regulation of bone resorption and formation during remodelling (Zheng et al. 1992). Similar to prostaglandins, certain cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) are potent stimulators of bone resorption. Pacifici et al. (1987) found that in disease states IL-1 released in peripheral monocyte cultures was increased in osteoporotic women with high bone turnover. Findings by Pfeilschifter et al. (1989) suggest that IL-1 and TNF-α act synergistically on the formation of osteoclasts. To determine this they exposed human bone marrow cell cultures to varying concentrations of both IL-1 and TNF-α and observed the number of osteoclast-like multi-nucleated cells formed. Their results indicated significant increases in osteoclast-like multi-nucleated cell formation when TNF-α and IL-1 were added together, suggesting that the extent to which these cytokines may stimulate bone resorption is largely dependent on their combined effects.

A later study by Cohen-Solal et al. (1993) also suggested a possible synergistic effect between IL-1 and TNF-α on bone. However, they measured the production of cytokines in the supernate of bone cultures obtained from pre- and post-menopausal women. Their results indicated a significant correlation between bone-resorbing activity, IL-1 and TNF-α levels in the supernates.

Vitamin E and Local Regulators of Bone Metabolism

Abundant research has focused on the ability of vitamin E to suppress inflammatory mediators. The activity of PGE_2, a product of the cyclooxygenase pathway,

is dependent on the cyclooxygenase enzyme (COX-2). The COX-2 enzyme requires the presence of oxidant hydroperoxide as an activator. Wu et al. (2001) hypothesized that vitamin E may suppress COX-2 activity by scavenging the oxidant hydroperoxide necessary for activation and thereby decreasing PGE_2 production. To investigate the effect of vitamin E on PGE_2 production, they used young (6 month) and old (24 month) male mice fed diets containing 30 ppm (recommended) and 500 ppm (supplemental) α-tocopherol for 30 days. At necropsy, peritoneal macrophages were collected and lipopolysaccaharide (LPS) stimulated PGE_2 production was measured in culture medium by radioimmunoassay. Their results indicated that macrophages from old mice in the control group produced significantly higher levels of PGE_2 compared to the young mice. However, vitamin E supplementation completely elimated the age-related increase in PGE_2 production. There was no significant difference between PGE_2 production in the young control group and old supplemented group.

Vitamin E has also been shown to suppress cytokine production. In two separate studies, Devaraj and Jialal (1996 & 2000), found that vitamin E supplementation significantly inhibited IL-1 and TNF-α production in LPS stimulated monocytes in both healthy and diabetic subjects. Furthermore, van Tits et al. (2000) also showed that vitamin E supplementation significantly inhibited the release of IL-1 and TNF-α in LPS stimulated mononuclear cells.

Oxidation and Bone Resorption

Many different hormones and biochemical factors are involved in bone remodeling; however, the precise signaling processes are poorly understood.

Abundant research has focused on the damaging effects of oxygen-derived free radicals. A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. The oxygen molecule, as it occurs naturally, is considered a free radical due to the presence of two unpaired electrons in its outer orbital. Osteoclasts are most likely derived from macrophages, which when stimulated, release O_2 into the surrounding tissue fluid. In the tissue fluid, O_2 promotes the formation of hydrogen peroxides and the hydroxyl radical (Halliwell & Gutteridge 1985).

Garrett et al. (1990) investigated whether the formation and activation of bone resorbing osteoclasts was associated with oxygen-derived free radical generation. The experiment was conducted not only in vitro using fetal rat long bones and mouse calvariae, but also in vivo. To determine the effect of oxygen-derived free radicals generated in vivo, a solution of xanthine and xanthine oxidase was injected into the subcutaneous tissue of the calvariae of 5 week old mice three times a day for 3 days. Histological data showed that the in vivo treatment of calvarial bone with xanthine and xanthine oxidase dramatically increased the number of osteoclasts and resorption surfaces in histologic sections. In vitro, nitroblue tetrazolium (NBT) staining of mouse calvaria was used as a marker of free radical production in resorbing bone. Bone resorption was stimulated by parathyroid hormone (PTH), IL-1α, and tumor necrosis factor (TNF). Microscopic examination found large, NBT-positive osteoclasts adjacent to areas of resorption, but few NBT-positive osteoclasts were seen in the control bones. They were able to confirm that when free oxygen radicals were generated in the bone environment, osteoclasts were formed, and bone resorption occurred.

Furthurmore, Key and collegues (1994) concluded that exposure of bone matrix to superoxide generated directly within the ruffled border resulted in bone resorption. They obtained cells from the tibiae of rats weighing approximately 135g and identified osteoclasts by staining for the presence of tartrate-resistant acid phosphatase (TRAP). Tartrate-resistant acid phosphatase positive cells were incubated in the presense of NBT, NBT plus superoxide dismutase (SOD), or NBT plus human recombinant calcitonin (hCT). Analysis of NBT staining in or around the osteoclasts was determined using microspectrophotometric densitometry with digitizing software. The greatest optical density was apparent in cells incubated in NBT alone. A significant, 1.84 fold reduction in NBT staining was observed in cells incubated with NBT and SOD when compared to those incubated in NBT alone. In these conditions, SOD does not cross the cell membrane, thus, any reduction in NBT staining is attributed to superoxide generated upon or diffusing to the external membrane of the osteoclast. Osteoclasts incubated with NBT and hCT also had a significantly reduced optical density when compared to controls. From this, they were able to conclude that superoxide is one of the oxygen-derived free radicals produced by osteoclasts and that the production of oxygen-derived free radicals is inhibited by SOD and hCT. A previous study by Key et al. (1990) supports the idea that exposure of bone to superoxide generated within the ruffled border results in increased resorption and that the addition of SOD significantly reduces the degradation of bone.

Vitamin E and Bone Metabolism

Vitamin E is a fat-soluble vitamin that functions to protect the membrane integrity of cells. This is achieved by vitamin E's ability to prevent the oxidation of unsaturated fatty acids contained in the phospholipids of cellular membranes (Knight 2000). Vitamin E acts as an antioxidant by reacting with lipid peroxy radicals to form a vitamin E radical. Vitamin E radicals are insufficiently reactive to abstract hydrogen ions from the membrane lipids. Therefore, vitamin E terminates the chain reaction of lipid peroxidation. Vitamin E radicals are fairly stable because the unpaired electron on the oxygen atom can be delocalized into the aromatic ring structure which increases its stability (Scarpa et al. 1984).

Because it has been shown that bone resorption is linked to free radical production (Garrett et al. 1990, Key et al. 1994) and that vitamin E suppresses oxidation, research has been conducted to determine the effect of vitamin E on bone metabolism.

Neve et al. (1993) examined the effect of vitamin E on bone physiology in both young and old, male and female CBA mice. In an 8 x 2 factorial design, animals were supplemented with 0.25% dl-alpha-tocopherol added to their usual pelleted diet. Young animals were necropsied at 7 weeks and old animals at 22 months. Femurs were removed for analysis. All animals in the supplemented group showed significantly higher bone mineral content in the upper half of the femur compared to those in the control group, regardless of sex or age. This study indicated a benefical effect of vitamin E on bone mass. However because oxidative stress was not measured in the mice, the researchers

were unable to determine if the beneficial effect was due to vitamin E's ability to prevent oxidative stress or by some other mechanism.

In another study, Xu et al. (1995) found that vitamin E supplemention in chicks significantly increased bone mineral apposition rates. Their study investigated the effect of vitamin E and and various lipids on epiphyseal growth plate cartilage development and trabecular bone formation. In a 2 x 2 factorial design, chicks were supplemented with 30 or 90 IU *dl*-α-tocopherol acetate/kg of diet. Thirty IU/kg served as the control diet and 90 IU/kg as 3 times the recommended level. Diets also contained either anhydrous butter oil (BSO), 40 g/kg diet, or soybean oil (SBO),100 g/kg diet. Animals consumed their respective diet for 14 days and were then necropsied. Chicks consuming 90 IU vitamin E/kg had significantly increased zonal thickness of the lower hypertrophic chondrocyte zone and increased growth plate thickness when compared to chicks consuming 30 IU/kg. The effect of vitamin E on labeling measures of trabecular bone was dependent on lipid source. Chicks consuming 90 IU/kg vitamin E with SBO had a higher total labeled bone surface and bone formation rate, than those consuming 90 IU vitamin E/kg with BSO or those consuming 30 IU vitamin E/kg with either lipid source. Their findings indicated that free radical production may not only increase osteoclastic activity, but also inhibit osteoblastic activity, making vitamin E vital for the protection of chondrocyte membranes and matrix vesicles from oxidation. They suggest that vitamin E may improve normal maturation and differentiation of chondrocytes and benefit matrix vesicle-initiated mineralization. In a related study examining the influence of vitamin E on metacarpal growth plate evolution and collagen characteristics in suckling lambs, Maiorano et al. (1999) found a significant vitamin E effect on thickness of the metacarpal growth plate

proportional to vitamin E dose. Their findings correlate with those of Xu et al. (1995) that vitamin E protects chondrocyte membranes from lipid peroxidation and inhibits cartilage resorption.

Assessment of Vitamin E Status in Rodents

The American Institute of Nutrition (AIN) rodent diets are nutritionally adequate, semi-purified, standardized diets formulated for growth, pregnancy and lactation, and maintaining the health of rodents during normal husbandry. The AIN-93G diet is recommended during growth, pregnancy, and lactation. However, the AIN-93M diet is lower in fat and protein, and is recommended for optimum nutrition during adulthood.

Fat source and amount have been shown to affect the vitamin E requirement in rodents. The AIN recommends increasing the amount of vitamin E when the amount of fat is increased in the diet (Reeves et al. 1993). Meydani et al. (1987) found that consumption of n-3 fatty acids reduced vitamin E concentrations in serum and tissues more than n-6 fatty acids. In an additional study (Meydani et al. 1988), they reported that fish oil moderated the increase in plasma α-tocopherol levels after vitamin E supplementation.

The AIN-93G diet contains 70 g soybean oil/kg of diet. The recommended amount of supplemental vitamin E for this source and amount of fat is 75 IU α-tocopherol acetate/kg of diet. Hypothesizing that vitamin E is beneficial in preventing age-associated lipid peroxidation and eicosanoid production, the AIN committee retained the amount of vitamin E in the AIN-93M diet at 75 IU α-tocopherol acetate/kg of diet as

in the AIN-93G diet. However, the amount of fat in the maintenance diet is only 40 g soybean oil/kg diet (Reeves et al. 1993).

Serum or plasma α-tocopherol is commonly measured to determine vitamin E status in rodents. Meydani et al. (1987) measured the serum α-tocopherol concentration in 3-month old male mice. Mice were fed a diet containing either 30 IU/kg or 500 IU/kg α-tocopherol acetate for 1.5 months. Tocopherol-stripped corn oil was used as fat source in the diets. Serum vitamin E, determined by high-performance liquid chromatography (HPLC), was 2.9±0.4 and 7.2±0.3 μg/ml in mice fed the 30 IU/kg and 500 IU/kg diets, respectively. Chen et al. (1995) measured the serum α-tocopherol concentrations in weanling, male rats fed 0, 100, 5000, and 15000 ppm vitamin E/kg diets for 2 months. Corn oil stripped of α-tocopherol was used as a fat source and supplied 14% total weight of diet. Serum α-tocopherol concentrations ranged linearly from 0.2±0.03 μg/ml for the 0 ppm α-tocopherol/kg diet to 13.6±2.1 μg/ml for the 15000 ppm α-tocopherol/kg diet. Additionally, Lehmann (1981) reported a linear increase in plasma α-tocopherol concentrations ranging from 1 to 7.5 μg/ml in male weanling rats consuming 0, 5, 10, 20, and 500 ppm α-tocopherol acetate/kg diet for 2.5 months.

Vitamin E deficiency is rare and no defined deficiency diseases have been established. However, increased susceptibility to oxidative damage has been associated with vitamin E deficiency. Hemolysis, measured in vitro using hydrogen peroxide as an oxidizer, is often used to measure vitamin E adequacy in subjects (Boda et al. 1998).

Studies have shown that excess α-tocopherol is not mutagenic, carcinogenic, or teratogenic in rats (Abdo et al. 1986, Dysmsza & Park 1975). However, rats fed

extremely high doses have experienced an assortment of other effects. At very high doses α-tocopherol may cause hemorrhaging and interfere with blood coagulation. Abdo et al. (1986) examined the effect of 13 weeks of *d*-α-tocopherol supplementation in 1 month old Fischer 344 rats. Vitamin E dosage (125, 500, or 2000 mg/kg body weight) was administered daily in corn oil by gavage. They found vitamin E to dose-dependently increase time required for blood to clot. Increases in prothrombin time were observed in animals receiving the 500 mg/kg α-tocopherol dose; however, prothrombin time was increased by 8.6 seconds in animals receiving the 2000 mg/kg dose when compared to animals receiving the 500 mg/kg dose. In a similar study, Yang and Desai (1977) examined the effects of diets containing 0, 25, 250, 2500, 10000, and 25000 IU α-tocopherol/kg diet in weanling Wistar rats. Prothrombin time was measured at 9, 12, and 16 weeks of supplementation. They observed no increase in prothrombin time even when dietary vitamin E was as high as 25000 IU/kg diet. Their results differed from those of Abdo et al. (1986) who found an increase in prothrombin time in animals consuming excessive amounts of α-tocopherol. However, because neither study measured plasma or tissue concentrations of vitamin E, it is difficult to assess the true vitamin E status of the animals.

Mechanical Loading and the Human Skeleton

Mechanical loading plays an important role in the achievement and maintenance of peak bone mass. Over a century ago, Julius Wolff hypothesized that "mechanical stress determined the form and function of bone" (Gooch 1997). Mechanical loading affects bone structure by enhancing the periosteal apposition rate during growth and

inhibiting age-related bone loss in adults. Strain or stress applied to bone enables osteoblasts to form new bone on existing surfaces (Parfitt 1987). Loading of bones is achieved through weight-bearing exercise and by hydrostatic pressures generated within the body. Regions of the skeleton which are exposed to the greatest weight-bearing load express a higher bone mineral density than areas exposed to less of a load (Iwamoto et al., 1999). Interstitial fluid pressures also increase the weight-bearing load on bones. Gravity creates a fluid pressure gradient from the top to the bottom of the body. Therefore, bones most affected by interstitial pressure are those of the lower body, such as the tibia and calcaneus (Turner 2000). Bailey et al. (1999) conducted a 6-year longitudinal study to investigate the effect of physical activity on bone mineral accrual in growing adolescents. Results were compiled from 68 boys and 72 girls, all of Caucasian descent. They found a greater peak bone mineral accrural rate and a 9% - 17% greater total body bone mineral content in children from the highest physical activity quartile compared to those in the lowest quartile. Their results further demonstrated that bone remodelling is affected by everyday physical activity.

Mechanical Unloading and the Human Skeleton

Mechanical unloading has many detrimental effects on the human skeleton. Unloading most often occurs in bedridden, or immobilized (paralysis) persons, and also in astronauts, who are exposed to microgravity.

Much of the information concerning the impact that unloading has on the human skeleton comes from studies examining astronauts. Reports on microgravity induced bone loss originated in the early 1970's, shortly after the first human space flights. The

Skylab flights of 1973 provided valuable information concerning the homeostasis of bone. Loss of bone density from the calcaneus was greater in astronauts aboard the longer flights than in those aboard the shorter flights. However, decreases in the bone mineral density of bones in the upper torso, such as the radius and ulna, were minimal (Vogel et al., 1977). More recently, Vico et al. (2000) measured the bone mineral density of the tibia and radius in fifteen cosmonauts aboard the Russian MIR space station. Length of stays ranged from 1 to 6 months. Bone mineral density (g/cm^3) was measured in the tibia and radius of the cosmonauts by a pQCT system called Densiscan. They found a 1.7% decrease in bone mineral density of the tibia after one month of spaceflight and 5.4% decrease after 6 months. By contrast, no significant reduction was seen in the radius at any flight duration. This evidence supports the hypothesis that bones exposed to higher levels of hydrostatic pressure and weight-bearing load are the most sensitive to increased resorption in the absence of stimulation.

During the 180 day Euromir 95 space mission, a study (Caillot-Augusseau et al., 1998) was conducted to measure the variations in biochemical markers of bone formation and resorption. Before, during, and after the flight, blood and urine samples were collected from four male cosmonauts for evaluation of serum alkaline phosphatase (ALP), osteocalcin (BGP), and type-1 procollagen (PICP) as markers of bone formation, and urinary pyridinoline excretion as a marker of bone resorption. Serum ALP, BGP, and PICP were decreased by 28%, 27%, and 38%, respectively, during the flight when compared to preflight values, and returned to normal approximately 7 days after landing. Conversely, total urinary pyridinoline in urine was increased by 35% during the spaceflight when compared to control values, indicating an increase in bone resorption.

Although a good model for study, space flights are generally limited in number of subjects and frequency. Therefore, ground-based models have been developed for examining the effect of unloading on the human skeleton. Currently, the most popular model is prolonged bedrest using a head down tilt to simulate cephalic fluid shifts. Studies using this model have provided data similar to that of spaceflight studies. Leblanc et al. (1990) reported that in six male subjects subjected to 17 weeks of bedrest, the most extensive bone loss occurred in bones of the lower body such as the calcaneus, while bone density increased in the skull. However, bone mineral density was found to remain normal in bones of the forearm.

To most effectively research the phenomenon of bone loss, a bone sample is necessary. However, with respect to experimental ethics, a bone biopsy is generally considered as being too invasive. Therefore, the only bone samples available for laboratory tests have been those obtained from animal models.

Mechanical Unloading and the Rat Skeleton

As an animal model, the rat has been used extensively in spaceflight research. Histomorphometric analyses on the skeleton of 2.7 month old rats flown aboard the 18.5 day COSMOS 1129 mission indicated an inhibition of periosteal bone formation in the tibia and humerus as compared to control rats. However, the decreased rate in the humerus was not as marked as in the tibia. The periosteal bone formation rate (10^{-3} mm^3/day) observed in the tibia of flight rats was 55% less than that of vivarium controls. Although not as severe, a 39% decrease in periosteal bone formation was found in the humerus of flight rats when compared to vivarium controls (Wronski & Morey, 1983).

Additionally, Vico et al. (1993) reported a 43% reduction in the primary spongiosa width of rats flown aboard the 14-day Cosmos 2044 flight as compared to vivarium controls suggesting altered longitudinal bone growth. Moreover, Patterson-Buckendahl et al. (1987), observed a a 22% decrease in serum osteocalcin of 2-month old, male rats subjected to a 7-day spaceflight. Third lumbar dry bone weight decreased by 17.5% in flight animals, while dry weight of the humerus decreased by 13% in flight animals compared to preflight controls. They also observed a decrease in the mineral content of the vertebrae and humerus of flight animals.

On earth, a hindlimb unloaded (HLU) model has been developed to study the impact of mechanical unloading on bone formation and resorption in rodents. This model enables researchers to examine in detail the physiological and cellular mechanisms of the skeletal response to mechanical loading.

Hindlimb Unloading and Bone Studies

In the HLU model, orthopedic traction tape, placed along the tail, is used to attach the rat to a pulley, elevating the hindlimbs and placing the rat in a 30° head-down angle. The head down tilt produces a cephalic fluid shift similar to space flight. Because the forelimbs are normally loaded, they can provide internal controls. The forelimbs also allow for ambulation, eating, and grooming (Morey-Holton and Globus 1998).

Globus et al. (1986) investigated the effect of hindlimb unloading on 2.5 month old rats. Rats were randomized into groups and suspended for 2-15 days, then necropsied and bones collected. Final body weight of rats unloaded for 15 days was not significantly different from controls, however it was reduced by 5%. After 15 days of skeletal

unloading, the dry weights of the tibia and L-1 vertebrae had decreased to 81% and 78% of the control values, respectively. Atomic absorption spectrophotometry was used to measure the calcium concentration in selected bones. The calcium concentration, expressed as milligrams of calcium to milligrams of fat-free weight, was significantly lower in suspended animals than in the control group, resulting in a lower percentage of mineralized bone in unloaded rats. However, changes in mineral concentration were not observed in the humerus or C-1 vertebrae suggesting minimal hormonal stress to the animal. Novikov and Ilyin (1981) also found decreases in the bone calcium concentration of rats subjected to hindlimb unloading. Their study examined the age related reactions of bones to unloading. Rats 1.5, 2.5, and 6 months old were suspended for 22 days and necropsied. Calcium concentrations in the proximal and distal epiphysis and the diaphysis of the femur were measured using flame absorption spectrophotometry. Animals in all three groups had significantly decreased calcium concentrations in the distal epiphysis as compared to the controls. In all three groups calcium concentration in the distal epiphysis decreased by 9%, but a significant decrease (9%) in calcium concentration of the proximal epiphysis was found only in rats aged 2.5 months. Although not significant, a 6% decrease in calcium concentration was observed in the proximal epiphysis of rats aged 1 and 6 months. No significant differences were found in calcium concentrations of the diaphyses in any groups. However, in this study, mineral concentration was only measured in the femur and not the normally loaded bones. In 5-month old female rats suspended for 28 days, Shaw et al. (1987) observed a non-significant 6% decrease in the calcium concentration of the femur compared to controls. The calcium concentration was measured fluorimetrically and expressed as micrograms

of calcium per milligram dry bone weight. Vailas et al. (1988) also fluorometrically measured the calcium concentration of the femur mid-diaphysis from 3.5 month old rats subjected to 28 days of hindlimb unloading. They found no significant differences in calcium concentration (μg/mg dry weight) between groups. However, calcium concentration was decreased by 3.5% in unloaded animals compared to controls. Similar final body weights were found between groups.

Furthermore, in 1991, Vico et al. compared bone histomorphometric data of 3.5 month old rats subjected to 7 days of spaceflight versus 7 days of hindlimb unloading. Final body weights of rats subjected to both spaceflight and hindlimb unloading were significantly less than body weights at baseline. Final body weight of rats in the spaceflight group was 9% less than the spaceflight controls and final body weight of hindlimb unloaded rats was 15% less than hindlimb unloaded controls. The mean thickness of the primary spongiosa and loss of trabecular bone was significantly higher in flight animals than in hindlimb unloaded animals. The decrease in trabecular density (43%) was also more dramatic in flight animals. However, an increased number of osteoclasts (113%) and active resorption surfaces (107%) were observed in suspended animals, but not in flight animals, suggesting that bone resorption as a result of hindlimb unloading may be mediated through the production of stress hormones. However, corticosterone levels were not measured in their study. Morey-Holton and Globus (1998) have concluded that in studies in which growing animals fail to gain weight and resorption is increased, stress may be a factor. To investigate this, Halloran et al. (1988) conducted a study to determine whether the inhibition of bone formation induced by skeletal unloading is a consequence of increased plasma glucocorticoids. Male, 125-175

gram Sprague-Dawley rats were hindlimb unloaded for 7 days. Ambulatory animals were pair fed and acted as controls. Plasma corticosterone was determined by radioimmunoassay on plasma collected every 6 hours for 24 hours on day 3 of unloading. No significant differences in plasma concentrations of corticosterones were found between groups. The 24-hour mean plasma concentrations of corticosterone were nearly identical in ambulatory (9.7 μg/dl) and hindlimb unloaded (9.4 μg/dl) animals. No significant difference in final body weight was observed between groups. The results suggest that inhibition of bone formation in skeletally unloaded rats is not a consequence of increased plasma glucocorticoids.

Mechanical Unloading and Bone Cell Cultures

Cell cultures have been used to examine what effect microgravity plays on bone directly at the cellular level. Carmeliet et al. (1997) examined the effect of 9 days at zero gravity on cells of the human osteosarcoma cell line MG-63 aboard the Foton 10 satellite. Cells were cultured in Dulbecco's modified eagle medium (DMEM) supplemented with fetal calf serum and antibiotics for 9 days, then detached with trypsin and replated. After treatment with Vitamin D_3 and TGF-β_2, cells were scraped, lysed in Tris (20mM) and sonicated. Alkaline phosphatase (ALP), protein, and DNA contentrations were measured on the lysate. Osteocalcin was also measured in the culture medium. They found a significant decrease in ALP activity of cells at microgravity compared to controls. Gene expression of collagen type 1, ALP, and osteocalcin was also significantly decreased at microgravity as compared to controls.

Cultured cell lines provide substantial information regarding the effect microgravity has on bone cells. However, primary cultures from marrow-derived cells have granted a better understanding of the uncoupling of bone remodelling that occurs during unloading.

Machwate et al. (1993) examined the effect of skeletal unloading on marrow-derived cells obtained from 1-month old male rats. Animals were hindlimb unloaded for 14 days, necropsied, and bone marrow collected from the right tibia. Collected cells were cultured in DMEM until preconfluency and detached with trypsin. As a marker of proliferation, cellular DNA synthesis was measured using [^{3}H]thymidine incorporation. Alkaline phosphatase activity was measured by scraping the cell layer into distilled water and sonicating cells to release ALP. Significantly decreased DNA synthesis, indicated by [^{3}H]thymidine incorporation was observed in cells of unloaded animals, but not controls. The ALP activity measured in bone marrow cells was not significantly different between hindlimb unloaded and control animals. In a similar study using 1-month old male rats immobilzed via sciatic neurectomy, Keila et al. (1994) found reduced ALP activity in cells from immobilized rats; however, [^{3}H]thymidine incorporation was not significantly different between immobilized and sham-operated animals. These inconsistencies may be attributed to different techniques and methods used for culturing the cells.

Indicators of Bone Formation

Osteoblasts are cells responsible for bone formation. Osteoblastic cells are rich in the enzyme alkaline phosphatase (ALP), which is often used as an indicator of bone formation.

In response to mechanical unloading, Machwate et al. (1993) found a significant decrease (21-24%) in the serum ALP concentration of rats subjected to a 14-day tail-suspension as compared to a control group. In a similar study, Keila (1994) also found a significant decrease in ALP positive marrow cells in rats unloaded by sciatic neurectomy.

Osteocalcin is also often measured as an indicator of bone formation. In 1985, Delmas et al. correlated serum osteocalcin levels to indices of bone turnover in humans. Synthesized by the osteoblast, osteocalcin is the most abundant noncollagenous protein in bone. To become functional, osteocalcin is dependent on vitamin K for the gamma-carboxylation of its three glutamate residues to form gamma-carboxyglutamic acid (Gla). In the absence of adequate vitamin K, not all of the Gla residues are carboxylated, which decreases its function (Booth 1997). Caillot-Augusseau et al. (2000) measured the concentration of undercarboxylated osteocalcin (Uoc) in the serum of two males, *collected prior to, during, and after* a 21-day spaceflight. They found a 23% increase in serum Uoc after 8 days of spaceflight, that decreased to preflight concentrations upon landing. Their results suggested that decreases in the carboxylation of osteocalcin may be correlated with spaceflight associated osteopenia.

In a study examining the effect of a 14-day tail-suspension on plasma osteocalcin levels, Machwate et al. (1993) found a 29% decrease in plasma osteocalcin of unloaded animals compared to ambulatory controls. Similarily, Patterson-Buckendahl et al. (1989) found a significant decrease (20%) in serum osteocalcin, measured by radioimmunoassay, of unloaded animals after 5 days of suspension. However, values returned closer to normal after 15 days suggesting that unloading and not environmental stress affected bone formation.

Indicators of Bone Resorption

Acid phosphatase is one of several enzymes produced by osteoclasts to aid in the digestion of bone matrix constituents (Marks and Popoff 1988). Studies have demonstrated that an effective way of differentiating osteoclasts from other endosteal cells is by examining the presence of acid phosphatase activity, which shows resistance to inhibition by sodium tartrate (Hammerstrom et al., 1971).

It has been demonstrated that bone resorption in vitro results in greater concentrations of acid phosphatase in culture media (Susi et al. 1966). Kalu (1990), investigated the effect of ovariectomy on the proliferation of tartrate resistant acid phosphatase (TRAP) cells in cultures of bone marrow cells from ovariectomized and sham operated rats. Marrow cells were harvested and cultured in α-minimal essential medium for 8 days. After 8 days, cultures were terminated and plates were stained for TRAP-positive cells using a commercially available kit. His results indicated that overiectomized rats form significantly more TRAP-positive cells in culture than sham-operated rats.

CHAPTER III

MATERIALS AND METHODS

Animals and Diets

Using a 2x3 factorial design, ninety-seven, male Sprague-Dawley rats (Harlan Teklad, Indianapolis, In) were randomly placed on a diet of 15, 75, or 500 IU *dl*-α-tocopherol/kg diet and assigned to hindlimb unloaded (HLU) or control (ambulatory) groups. The diet was based on a diet recommended for rodents (AIN-93M, powder). Diets were prepared by Harlan Teklad and contained tocopherol stripped soybean oil as a fat source (40g/kg diet). Animals were separated into 3 replications (n=25, n=35, n=36) due to a limited number of cages adaptable to hindlimb unloading. The replicate groups were obtained so that all rats were approximately 11 months of age at the time of unloading. The animals were individually housed and kept in an environmentally controlled laboratory at the Oklahoma State University Laboratory Animal Resource Center (LAR). Animals were maintained on a 12:12 hour light/dark cycle and had ad libitum access to deionized water at all times during the study. The project was approved by the Institutional Animal Care and Use Committee at OSU, protocol #733 (Appendix A). Guidelines for the ethical care and treatment of animals established by the committee were strictly followed.

After arriving at the housing unit, rats were initially fed a diet of standard chow and allowed to acclimate to the environment for several days. They were then fed the AIN-93M powdered diet containing no vitamin E for 10 days. After the 10 day period, rats were placed on their assigned vitamin E diet (low, recommended, or high), which

was consumed for 2 months. At this point rats were sedated with an intraperitoneal injection of ketamine (50 mg/kg body weight) and xylazine (2.5 mg/kg body weight) for full body scanning on the DEXA, tail blood was collected, and animals assigned to the hindlimb unloading group were placed in suspension according to the method of Wronski and Morey-Holton (1979). The tails of animals to be unloaded were cleaned using isopropyl alcohol and coated with tincture of benzoin. A 1-cm wide strip of orthopedic tape was attached laterally along each side of the tail to form a loop near the end of the tail. The tail was then wrapped with a cotton gauze and secured with 3 strips of tape. The loop was attached to a pulley system allowing the hinquarters of the animal to be elevated, placing the animal in a 30° head-down hilt. The animals maintained free movement about the cage using their forelimbs. Rats continued to consume their assigned diets for the 28 day suspension period. Ambulatory rats acted as controls and were pair-fed their respective diets according to the food consumption of the hindlimb unloaded animals during replication #1. For replications #2 and #3, feeding of the ambulatory animals was based on consumption data from the first replication.

Necropsy

After 28 days of suspension, hindlimb unloaded and control rats were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight), and exsanguinated from the abdominal aorta. Collected blood was allowed to clot and centrifuged at 1500 RPM for 20 minutes at 4°C to separate serum. Aliquots of serum were frozen and kept at -20°C unil further analysis.

Femur, tibia, humerus, and vertebral columns were collected from animals and carefully cleaned of adhering tissue without removal of the periosteum. The vertebral column was first frozen at -20°C and lumbar vertebrae were separated and cleaned of all adhering tissues at a later date. Cleaned bones were stored at -20°C until further analysis.

Isolation of Bone Marrow Cells

At necropsy, the right tibia of each animal was carefully removed, cleaned of all soft tissues, and dipped briefly in 95% ethanol before being placed in 0.01M sterile phosphate buffered saline (PBS) (Sigma P-4417) supplemented with 2% antibiotics (10,000 units penicillin and 10 mg streptomycin in 9% NaCl, Sigma P-0781). Tibias were processed using sterile techniques in a laminar flow hood. The epiphyseal area of each tibia was carefully removed with bone cutters and cut transversely with a scalpel. Bone marrow was then flushed out with PBS and collected marrow cells were centrifuged in a sterile tube for 10 minutes at 1000 RPM and room temperature. Supernate was discarded and cells pellets were resuspended in bicarbonate-buffered α-Minimum Essential Medium (α-MEM)(Sigma M-0644), containing 10% heat-inactivated fetal bovine serum (FBS)(Gibco 16000-004), 5 X 10^{-8} M Vitamin D_3 (Sigma), and 2% antibiotics. Suspended cells were passed through a cell strainer to remove marrow debris.

Cell viability was assessed using the trypan blue exclusion technique. Approximately 100 µl of cell solution was added to 100 µl of trypan blue (Gibco 15250) and allowed to sit for 5 minutes. The cell suspension was placed into a hemacytometer and viable and non-viable cells were counted for 10 squares. Viable cells were identified as those not taking up the blue dye. Viable cell concentration was calculated as: cells/ml = average viable cell count/square x dilution factor x 10^4. Total number of cells was calculated as: total cells = cells/ml x original volume. After assessing viability and cell concentration, cells were plated in 24-well plates at 0.5 X 10^6 cells/cm^2 with 8-12 wells per rat. Cultures were incubated at 37°C in a humidified atmosphere with 95% air and 5% CO_2. Media was changed every third day by carefully aspirating off spent media and replacing it with 0.5 ml of fresh media, warmed to 37°C. Media changes continued until cells reached pre-confluency at 2 weeks. Confluency was determined by cellular coverage in the well; preconfluency was designated as 80-90% coverage. Pictures were taken periodically throughout cellular differentiation and growth.

When cells reached pre-confluency, plates were centrifuged for 10 minutes at 1000 RPM and room temperature. Supernate from each well was collected into a 15ml conical tube on ice, and then aliquoted into microcentrifuge tubes and frozen at -20°C for furthur analyses of alkaline phosphatase and osteocalcin.

To measure the alkaline phosphatase activity (ALP) of lysed cells, 0.5 ml PBS was added to 2 wells from each animal and wells were sonicated (Sonics & Materials, Inc., VC-300 Sonicator, Danbury, CT) for 30 seconds. Cell lysate was collected into microcentrifuge tubes on ice and immediately frozen at -20°C until further analysis.

After collection of supernates, 4 wells from each animal were stained for tartrate-resistant acid phosphatase (TRAP) activity using a commercially available kit obtained from Sigma (387-A). TRAP positive cells were identified as those staining positive for tartrate resistance with 3 or more nuclei.

Biochemical Analyses

Serum and cell lysate ALP activity was measured on the COBAS Fara II clinical analyzer by a colorimetric method using a commercially available kit from Roche Diagnostics (Roche Diagnostic Systems, Indianapolis, IN). Alkaline phosphatase hydrolyzes 4-nitro-phenylphosphate to the 4-nitrophenoxide ion (Tietz 1987). The 4-nitrophenoxide ion has a strong absorbance at 405nm which is proportional the ALP activity in the sample.

Serum tartrate-resistant acid phosphatase (TRAP) was also determined colorimetrically using a kit obtained from Roche Diagnostics. For this measurement, α-naphthylphosphate is hydrolyzed by acid phosphatase to α-naphthol, which combines with Fast Red TR (diazotized-2-amino-5-chlorotoluene) to produce a dye which absorbs at 405 nm (Babson et al. 1959, Hillman 1971). The increased rate in absorbance is proportional to the acid phosphatase activity in the sample. When L-tartrate is added to

the reagent, acid phosphatases resistant to tartrate are inhibited, but all other acid phosphatases in the sample are absorbed at 405 nm.

For determining antioxidant capacity, the ferric reducing ability (FRA) was measured. The procedure developed by Benzie and Strain (1996) for determining FRA is performed on the clinical analyzer. At low pH's, antioxidants will cause a ferric-tripyridyl triazine (Fe^{3+}-TPTZ) complex to be reduced to the ferrous (Fe^{2+}) form. The Fe^{2+} forms an intense blue color with a 593 nm maximum absorption. Intensity of absorption is proportional to the total antioxidant capacity in the sample.

Serum osteocalcin and cell culture supernate osteocalcin were measured using a rat specific immunoradiometric assay (IRMA) kit obtained from Immutopics (Cat # 50-1500, San Clemente, Ca). In this assay serum or cell culture media is incubated with an antibody coated bead and osteocalcin contained in the sample is immunologically bound to the bead and forms a sandwich complex. After incubation, the bead is washed to remove any unbound labeled antibody and the radioactivity bound to the bead is measured using a gamma counter (Packard Cobra II, Downers Grove, Il). Radioactivity of the antibody complex bound to the bead is directly proportional to the amount of rat osteocalcin in the sample.

Serum vitamin E concentration of samples collected prior to suspension and at necropsy was determined using high performancex liquid chromatogrophy (HPLC). The method used was modified from Ortega et al. (1998) and Bieri et al. (1979). Standard solutions were prepared in 25, 50, and 100 μg/ml concentrations from α-tocopherol (Sigma, T-3251) dissolved in 95% ethanol (ETOH). Internal standard (5000 μg/ml) was prepared by dissolving α-tocopherol acetate (Sigma, T-3376) in ETOH. Serums were mixed with internal standard (200 μl) and vitamin E was extracted using hexane. Samples were centrifuged and the hexane layer carefully removed into a microcentrifuge tube and evaporated under nitrogen. Vitamin E was then redissolved by the addition of methanol (75 μl) and diethyl ether (25 μl). Ten μl's of each sample was

injected in duplicate into a C-18 column (Supelco, Bellfonte, Pa). A UV detector (Waters Tunable Absorbance Detector, Milford, Ma) was used with wavelength set at 290 nm. Mobile phase was a methanol:water mixture (95:5) at a flow rate of 2 ml/minute. Run time for the analysis was 16 minutes and retention time for α-tocopherol, 9 minutes and α-tocopherol acetate, 13 minutes.

Serum and urinary corticosterone were measured by radioimmunoassay with a kit obtained from Diagnostic Products Corporation (Catalog #TKRC1). In this assay rat corticosterone competes with corticosterone in the sample for antibody sites on the polypropylene tube. The radiolabeled corticosterone bound to the tube is then counted in a gamma counter to obtain a number which reflects corticosterone present in the sample.

Bone Analysis

Each right femur and third lumbar vertebrae was thawed and placed in a weighing boat. Bones were covered with approximately 2 cm saline solution and individually scanned by Dual Energy X-ray Absorptiometry (DEXA) (Hologic QDR 4500A, Waltham, MA.) using the small animal high resolution mode to determine bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD).

Femurs were fractured, marrow was removed, and bones were extracted for ALP and total bone protein (TBP). The procedure was based on the method of Farley et al. (1992). Alkaline phosphatase and TBP were extracted using a solution of 0.01% Triton X-100 with 0.02% sodium azide for 72 hours at 4°C. After incubation, tubes were centrifuged and extract decanted. Bone alkaline phosphatase was measured on the extract using the clinical analyzer and reagents from Roche Diagnostics. The mechanism of the reaction is listed above for the serum ALP. Total bone protein of the extract was determined by a biuret method on the clinical analyzer with a kit obtained from Roche Diagnostics. In this method divalent copper reacts with the peptide bonds of proteins

under alkaline conditions to form a biuret complex which absorbs at 540 mn (Henry et al. 1974).

To determine density by displacement, third lumbar vertebrae were soaked overnight in Type I water and then placed in a dessicator with vaccum for 1 hour. A density determination kit (Mettler ME-33340, Mettler Instruments, Grelfensee, Switzerland) was used to measure underwater weight and air weight. Density by displacement was calculated using Archimedes' principle.

Mineral Analyses

Third lumbar vertebrae and femurs were analyzed for mineral content. The bone samples were placed into pre-weighed, acid-washed tubes and dried in an oven for 24 hours at 100°C. Bones were cooled in the dessicator and dry weights recorded (dry weight). Bones were then wet and dry ashed according to a modification of the method of Hill et al. (1986). Blank tubes were prepared and carried throughout the procedure for calibration of the machine. Bone samples were placed in a heating block at 105°C and wet ashed using concentrated double distilled nitric acid (GFS Chemicals, Columbus, OH), 30% hydrogen peroxide (JT Baker, Phillipsburg, NJ) and Type I water. After bone fragments disintegrated, hydrogen peroxide was periodically added until bubbling ceased and samples were white in color. Tubes were left in the heating block until all liquid had evaporated and then placed in a muffle furnace at 375°C for 48 hours. Wet and dry ashing was repeated until bones were completely ashed. Weights were recorded when samples were completely ashed (ash weight).

After bones were fully ashed, the mineral residue was dissolved into a solution of 1.5 ml concentrated double distilled nitric acid and 1.5 ml distilled water (stock solution). Stock solutions were further diluted for specific mineral analysis using 0.05% double distilled nitric acid for magnesium, iron, copper, and zinc. For calcium determination, stock solutions were diluted with a solution of 0.5% nitric acid and 0.5 % lanthanum

chloride. Mineral analyses were performed using flame atomic absorption spectroscopy with an air-acetylene flame and deuterium background correction (Perkin Elmer Model 5100PC Atomic Absorption Spectrophotometer, Norwalk, CT) at the most sensitive wavelength for each element.

Statistical Analyses

Data analysis involved computation of means and standard error of the means for each of the treatment groups using SAS (version 8.0. SAS Institute, Cary, NC). Analysis of variance and least square means were calculated using the general linear model procedure and the means were compared using the least significant difference between groups. The differences were considered significant at $p<0.05$.

CHAPTER IV

HINDLIMB UNLOADING AND VITAMIN E INFLUENCE BONE COMPOSITION IN AGED SPRAGUE-DAWLEY RATS

Tracy L. Riggs, Brenda J. Smith, Sloan Martin, Bahram Arjmandi, Edralin Lucas, and Barbara J. Stoecker

Department of Nutritional Sciences, College of Human Environmental Sciences

Oklahoma State University

Stillwater, Oklahoma

ABSTRACT

The effects of hindlimb unloading, vitamin E, and their interaction on bone were investigated. Using a 2 X 3 factorial design, ninety-seven, 11-month old, male, Sprague Dawley rats were either hindlimb unloaded (HLU) or remained ambulatory (AMB) as controls. After 10 days on a diet containing no vitamin E, rats in each group were randomly assigned to one of three dietary treatments containing 15, 75, or 500 IU *dl*-α-tocopherol/kg diet, which was consumed for the 2 months prior to unloading. After 28-days of HLU, rats were necropsied and bones were collected for determination of bone mineral density and mineral concentrations. Selected biochemical measures of bone formation and resorption were also performed. Serum vitamin E was dose-dependently

elevated ($p<0.0001$) by dietary vitamin E treatment. Bone resorption, as indicated by serum tartrate-resistant acid phosphatase, was elevated ($p<0.0001$) in unloaded animals, but was not affected by diet. Serum and bone alkaline phosphatase and serum osteocalcin, markers of bone formation, were not significantly affected by diet or unloading. Hindlimb unloading significantly reduced bone protein extracted from the femur. Bone mineral density, measured by dual-energy x-ray absorptiometry (DEXA), of the third lumbar vertebrae (L3), femur, and humerus was significantly reduced by HLU, but not by diet. HLU significantly reduced iron concentration in the femur of unloaded animals; furthermore, HLU significantly decreased the total content of calcium, magnesium, iron, and zinc in the femur. In L3, HLU significantly reduced the total content of calcium, magnesium, copper, iron, and zinc. Diet had no effect on mineral in the femur or L3, except that, the L3 of animals on the 75 IU diet had higher total ($p<0.0172$) bone iron when compared to animals on the 15 IU and 500 IU diets.

These findings suggest that the osteopenia associated with hindlimb unloading in aged rats is a result of increased bone resorption and not diminished formation. However, we were unable to establish any protective effect of these doses of vitamin E on bone in this model. Further studies are needed to investigate the increased bone resorption observed in HLU aged rats, and to investigate the possible protective effect of vitamin E on bone.

INTRODUCTION

As the skeleton ages, the rate of bone resorption tends to exceed the rate of formation, resulting in a net loss of bone (Parfitt et al.1983). Although many factors are involved, a major cause appears to be a decrease in mechanical loading and usage (Bagi et al.1993). Loading of bones is achieved through weight-bearing exercise and by hydrostatic pressures generated within the body. In the absence of mechanical stimuli, regions of the skeleton exposed to the greatest weight bearing load suffer a more dramatic bone loss than areas with a smaller load (Iwamoro et al. 1990). Much of the information concerning what impact unloading has on the human skeleton comes from studies examining astronauts. Reports on microgravity-induced bone loss originated in the early 1970's, shortly after the first human space flights. Studies indicated significant losses of bone density from bones normally exposed to the greatest weight bearing loads, while bone loss was minimal from bones with normally low weight bearing loads (Vogel et al. 1977, Vico et al. 2000). Similar results have also been found in studies using prolonged bed rest with a head down tilt to simulate cephalic fluid shifts (Leblanc et al. 1995). As an animal model, the rat has been used extensively in spaceflight research. However, the development of ground based models has been promoted by extensive costs and limited space aboard flights. On earth, the hindlimb unloading model has proven useful to study the impact of weightlessness on bone. In this model, the head down tilt produces a cephalic fluid shift similar to space flight and, because the forelimbs are normally loaded, they can provide internal controls (Morey-Holton & Globus 1998). Hindlimb unloading has been shown to induce a reduction in the mineral apposition rate of young growing animals (Machwate et al. 1993). However, what effect hindlimb unloading plays on

mineral concentrations of bone is not well understood. Globus et al. (1986) and Novikov and Ilyin (1981) reported decreased bone calcium concentrations in hindlimb unloaded animals, while Shaw et al. (1987) and Wronski and Morey (1983) have observed minimal differences in calcium concentrations of unloaded bones. These inconsistencies may be attributed to differences in the procedures used to determine minerals, age or sex of animals, and length of unloading period.

Another factor influencing bone resorption is the presence of oxygen-derived free radicals. Research has shown that production of free radicals in the bone environment leads to osteoclast activation and subsequent bone resorption (Garrett et al. 1990, Key et al. 1994). Recent studies examining the effect of supplemental antioxidants, most notably vitamin E, on bone quality, have indicated a possible protective effect (Maiorano et al.1999, Neve et al. 1993, Xu et al. 1995). Considerable research has been conducted concerning vitamin E and its benefits against oxidative stress. However, very little research has focused on vitamin E and bone physiology.

This study was conducted to determine if hindlimb unloading, vitamin E, or their interaction affect bone density or selected biochemical markers of bone metabolism in aged rats. We also investigated the changes in concentrations of selected macro and trace elements in the unloaded rats.

MATERIALS AND METHODS

Animals and Diets

Using a 2 X 3 factorial design, ninety-seven, male Sprague-Dawley rats (Harlan Teklad, Indianapolis, In) were randomly fed a 15, 75, or 500 IU *dl*-α-tocopherol/kg diet

and assigned to hindlimb unloaded or control (ambulatory) groups. The diets (Harlan Teklad) were based on diet recommendations for rodents (AIN93M) (Reeves et al.1993) and contained tocopherol stripped soybean oil as a fat source (40g/kg diet). The animals were individually housed in an environmentally controlled laboratory. Animals were maintained on 12:12 light/dark cycles and allowed free access to distilled water throughout the study. Guidelines for the ethical care and treatment of animals established by the Animal Care and Use Committee at OSU were strictly followed.

After a 10-day initial washout period on a diet with no vitamin E, animals then consumed their assigned diet for the 2 months prior to unloading. Hindlimb unloading was performed according to the method of Wronski and Morey-Holton (1979). Orthopedic tape, placed laterally along each side of the tail, was used to attach a clip near the end of the tail. The clip was attached to a pulley system allowing the hindquarters of the animal to be elevated, placing the animal in a 30° head-down tilt. The animals maintained free movement about the cage using their forelimbs. Rats continued to consume their assigned diets for the 28-day unloading period. Ambulatory rats acted as controls and were pair-fed their respective diets.

Necropsy

After 28 days of suspension, animals were anesthetized intraperitoneally with ketamine (90mg/kg body weight) and xylazine (5mg/kg body weight) and exsanguinated from the abdominal aorta. Collected blood was allowed to clot, centrifuged, and serum aliquots frozen at -20°C until further analysis. The right femur, right humerus, and third lumbar vertebrae were collected from animals, cleaned of adhering tissues, and frozen at -20°C until further analysis.

Biochemical Analysis

Serum alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) were measured on the COBAS Fara II clinical analyzer using the appropriate reagents from Roche Diagnostics (Roche Diagnostic Systems, Indianapolis, IN). Ferric reducing ability (FRA) was measured on the clinical analyzer according to the method of Benzie and Strain (1996). Serum osteocalcin was measured using a rat specific IRMA kit obtained from Immutopics (Cat # 50-1500). A rat corticosterone kit (Diagnostic Products Corporation, Catalog #TKRC1) was used to measure both serum and urinary corticosterone levels by radioimmunoassay. Vitamin E concentration was determined using high precision liquid chromatography (HPLC) on blood serum collected prior to unloading and at necropsy according to the methods of Ortega et al. (1998) and Bieri et al. (1979). Vitamin E was extracted from serum using hexane and samples were injected into a C-18 column (Supelco, Model# 504971, Bellfonte, PA). A UV detector (Waters Tunable Absorbance Detector, Milford, MA) was used with wavelength set at 290 nm. Mobile phase was a methanol:water mixture (95:5) at a flow rate of 2 ml/minute.

Bone Analysis

Right femur, right humerus, and third lumbar vertebrae were thawed and then individually scanned by Dual Energy X-ray Absorptiometry (DEXA) (Hologic QDR 4500A, Waltham, MA) to determine bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD).

Prior to ALP extraction, femurs were fractured by another graduate student for a different aspect of this project. Fractured femurs were then extracted for bone ALP and

total bone protein using a solution of 0.01% Triton X-100 with 0.02% sodium azide, according to the method of Farley et al. (1992). Extract ALP was measured on the clinical analyzer in the same manner as serum ALP, described above. Total bone protein of the extract was determined by a biuret method with a kit obtained from Roche Diagnostics.

Third lumbar vertebrae and femur were ashed using a series of wet and dry ashing steps according to a modification of the method of Hill et al. (1986). After bones were completely ashed, the mineral residue was solubilized and analyzed for calcium, magnesium, iron, copper, and zinc using a flame atomic absorption spectrophotometer with an air-acetylene flame and deuterium background correction (Perkin Elmer 5100PC AAS, Norwalk, CT).

Statistical Analyses

Data analysis involved computation of means and standard error of the means for each of the treatment groups using SAS (version 8.0, SAS Institute, Cary, NC). Analysis of variance and least square means were calculated using the general linear model procedure and the means were compared using the least significant difference between groups. The significance level was set at $p<0.05$.

RESULTS & DISCUSSION

Vitamin E status was determined by measuring serum α-tocopherol concentrations prior to unloading and after the 28-day unloading period. Serum vitamin E concentrations were dose-dependently affected by dietary vitamin E concentrations, both prior to unloading and after as evidenced by a significant difference ($p<0.001$) among

vitamin E groups (Table 1). After 2 months of diet consumption, serum α-tocopherol concentrations in the animals consuming 75 IU and 500 IU α-tocopherol/kg diet were increased by 84% and 189% respectively, when compared to the animals consuming a 15 IU/kg α-tocopherol diet.

Although numerically smaller, similar percentage differences between vitamin E groups have been reported by Meydani et al. (1988) and Lehmann (1981). In 1987, Meydani et al. reported that α-tocopherol concentrations, in mice fed a 500 IU/kg diet, were 148% higher than concentrations in mice fed a 30 IU/kg diet. In our study, the α-tocopherol concentrations of animals consuming the 15 IU/kg and 75 IU/kg diets appeared to increase with time as evidenced by a 46% increase in serum α-tocopherol, between baseline (2 months on diet) and necropsy (3 months on diet) in animals on the 15 IU/kg diet, and a 25% increase in animals on the 75 IU/kg diet. However, serum α-tocopherol concentration in animals on the 500 IU/kg diet increased only by 8%, suggesting a possible plateau effect at high levels. A plateau effect, at approximately 3 to 4 times the unsupplemented concentration, has been observed in humans consuming large amounts of supplemental α-tocopherol. (Devaraj et al.1997, Jialal et al. 1995).

The final body weight of unloaded animals was significantly lower ($p<0.001$) than that of ambulatory animals; however, α-tocopherol in the diet had no effect on final body weight (Table 2). The weight loss as a result of hindlimb unloading in this study differs from the results obtained from studies examining young growing rats (Globus et al. 1986, Halloran et al. 1988). Although Globus et al. (1986) reported no significant difference in final body weights of 2.5 month old rats unloaded 15 days as compared to controls, the final body weight of unloaded animals was 5% less than that of controls.

The final body weight of unloaded rats in our study was only 6% less than that of controls. These statistical inconsistences may at least be partially attributed to a smaller number of animals in the studies with insignificant weight differences. The studies by Globus et al. and Halloran et al. involved ≈60 and 42 rats, respectively. The final number of rats in our study was more than 90 animals. Furthermore, patterns of weight loss in mature animals, similar to the ones we observed, have been reported in other studies (Vico et al. 1998, Smith 1999).

The question of what role stress plays on the body weight of unloaded animals remains unclear. Halloran et al. (1988) reported that the inhibition of bone formation in young hindlimb unloaded animals was not a consequence of increased plasma glucocorticoids or increased sensitivity to glucocorticoids. To examine this in our study, we measured both serum and urinary corticosteriod levels. Serum corticosteroid concentrations reflected no significant difference between the AMB and HLU groups (Table 3) and was measured in only 32 of the animals. Urinary corticosterone was also measured and reflected no significant differences between HLU and AMB animals. The kit used for urinary corticosterone was developed for the measurement of serum corticosterone and normal levels for urinary corticosterone were not available.

In this model, the humerus provides an internal control for distinguishing between local and systemic responses to hindlimb unloading. Ideally, no significant differences in bone mineral density would be observed between the humerii of unloaded and ambulatory animals. However, one might hypothesize that bone mineral density in the humerus would increase during hindlimb unloading. During hindlimb unloading, the 30° head down tilt imposed on animals increases the hydrostatic pressure in the forelimbs.

Interstitial fluid pressures increase the weight-bearing load on bones, and thereby, increase bone mineral density (Turner 2000). Observed bone mineral density decreases in the humerii of unloaded animals would suggest the bone loss associated with hindlimb unloading was at least somewhat the result of a systemic response to suspension (such as stress) and not only a result of the actual lack of weight-bearing activity. In our animals, we estimated the bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD)(g/cm^2) of the humerus using dual-energy x-ray absorptiometry (DEXA). We observed a 3% decrease ($p<0.0001$) in the humoral bone mineral density of unloaded animals compared to the control, but the level of vitamin E in the diet did not effect BMA, BMC, or BMD (Table 4). These results differ from those obtained in other HLU studies that observed no significant differences in the humoral bone mineral density of unloaded and ambulatory rats (Globus et al. 1986, Smith et al. 1999).

Femur dry weight and femur ash weight were significantly reduced by hindlimb unloading, however, no diet effect was observed (Table 5). An interaction between diet and hindlimb unloading was observed in femur BMA. Femur BMA in ambulatory animals on the 75 IU/kg diet was significantly greater than femur BMA of hindlimb unloaded animals on the 75 and 500 IU/kg diets. Hindlimb unloading significantly reduced femur BMC and BMD by 6.5% and 3.9%, respectively. Diet had no effect on BMC or BMD. For comparison, in cosmonauts, a 1.7 % decrease in tibial BMD was observed after one month of spaceflight, which increased to 5.4% after 6 months in space (Vico et al. 2000).

Neither HLU nor diet created any significant changes in femur mineral concentrations of calcium, magnesium, copper, or zinc, however HLU decreased

($p<0.009$) the mineral concentration of iron in unloaded rats, compared to controls (Table 6). Moreover, HLU significantly decreased the femoral total mineral content of calcium, magnesium, iron, and zinc, but not total copper content (Table 7). These results are in agreement with those of Valias et al. (1988) and Shaw et al. (1987) that 28 days of HLU does not decrease calcium concentration in the femur. The 15.3% decrease in femur iron concentration is perplexing. Prior to ashing, bone marrow was rinsed from the femurs to prevent mineral in the marrow from affecting the samples. The decrease could possibly suggest that iron was being removed from bone matrix for utilization elsewhere in the body. Hindlimb unloading did not significantly decrease total copper content of the femur. Copper is involved in collagen crosslink formation (Siegel 1978). If other constitutents of bone mineral were depleted, a decrease in copper would also be expected. Our results may possibly be attributed to the procedure used for measuring copper content. Concentrations of copper in the sample were very close to the detection limits of flame atomic absorption spectrophotometry and the resulting variability of the samples was high. A more sensitive measure, such as furnace atomic absorption spectrophotometry, may have reduced variability and detected a more significant depletion of copper.

Hindlimb unloading significantly reduced third lumbar dry weight and ash weight (Table 8). Diet did not affect third lumbar dry weight, but a nearly significant ($p<0.056$) diet effect was observed in third lumbar ash weight (Table 8). Animals assigned to the 75 IU/kg vitamin E diet had the greatest ash weight, while animals assigned to the 15 IU/kg diet had significantly less ash weight than the 75 IU group. Ash weight for the 500 IU/kg vitamin E was intermediate and did not differ from either the 15 or 75 IU/kg diets

(Table 7). Third lumbar BMA was not significantly affected by HLU or diet, however, BMD was significantly reduced in HLU animals (Table 8). A diet and HLU interaction was observed in L3 BMC. Third lumbar BMC was significantly less in hindlimb unloaded animals compared to ambulatory, regardless of diet. Ambulatory animals on the 75 IU/kg diet had a significantly greater BMC compared to ambulatory animals on the 15 and 500 IU/kg diets, however, no difference in BMC was observed between ambulatory animals on the 15 or 500 IU/kg diets. In addition, no significant diet effect was observed in the BMC of HLU animals on the 15, 75, or 500 IU/kg diets. Femur BMD and L3 BMD were positively correlated ($r=.68$, $p<0.001$). Interestingly, decrease in BMD from the third lumbar (10.3%) was more than double that of the femur (3.9%). This may be explained, in part, by bone biology. The high percentage of trabecular bone in the vertebrae is more sensitive to changes in bone turnover than the predominately cortical bone of the femur (Baron 1999). However, in theory, because the femur is normally loaded at a greater amount than the vertebrae, it would be expected to be more sensitive to hindlimb unloading. These findings may corroborate that bone loss occurring during unloading, in aged rodents, is more the result of systemic factors or cell to cell interactions such as cytokines and not necessarily unloading or related fluid shifts.

In the L3, hindlimb unloading significantly reduced the mineral concentrations of calcium and zinc, but did not affect magnesium or iron (Table 9). A HLU and diet interaction ($p<0.0258$) was observed in L3 copper concentration. Ambulatory animals on the 500 IU vitamin E diet and HLU animals on the 15 IU diet had significantly lower L3 copper concentration than HLU animals on the 500 IU diet. Diet did not affect the L3 mineral concentrations of calcium, magnesium, or zinc; however, iron concentration was

higher ($p<0.0172$) in animals assigned to the 75 IU/kg vitamin E diet compared those assigned to the 15 IU and 500 IU vitamin E/kg diets (Table 9). Total calcium, magnesium, copper, iron, and zinc contents in the L3 were significantly reduced by hindlimb unloading (Table 10). A diet effect was observed on total iron content of the L3. The 75 IU/kg vitamin E diet significantly increased the iron content of the L3 when compared to the 15 IU and 500 IU vitamin E diets.

In this study, markers of bone formation, alkaline phosphatase (ALP) and osteocalcin were measured in the blood serum collected at necropsy. Twenty-eight days of hindlimb unloading and vitamin E supplementation did not significantly affect either serum ALP or serum osteocalcin concentrations (Table 11). Bone ALP extracted from the femur was not significantly affected by diet or HLU, however it tended to be higher (11%) in HLU animals compared to AMB (Table 12). Extracted bone protein was reduced ($p<0.0103$) in HLU when compared to AMB, with diet having no effect (Table 12). Mechanical unloading has been shown to decrease ALP positive marrow cells in young growing rats (Machwate et al. 1993, Keila 1994), however ALP has not been previously examined in unloaded aged rats. Furthermore, Risteli and Risteli (1993) reported that ALP reached a maximum during matrix mineralization and then decreased as mineralization declined. Machwate et al. (1993) and Patterson-Buckendahl (1989) have reported decreases in serum osteocalcin of rats unloaded for short durations, but values returned closer to normal after 15 days of unloading. However, it should be noted that their observed osteocalcin concentrations (ng/ml) were more than 100 fold greater than osteocalcin concentrations in the present study. One possible explanation for the variation could be the dependence of osteocalcin on vitamin K to become activated.

Research has suggested that very high doses of α-tocopherol inhibit platelet aggregation and adhesion in vitro (Calzada et al. 1997, Freedman et al. 1996) and that the hemorrhagic effects can be reversed by administering supplemental vitamin K (Abdo et al. 1986). With this information, one might expect vitamin E to dose-dependently affect osteocalcin levels; however, we observed no difference in osteocalcin concentration by vitamin E group. Additionally, Machwate and Patterson-Buckendahl were measuring osteocalcin in a young animal, undergoing rapid bone development, while we were examining a mature animal at or near its peak bone mass.

Hindlimb unloading significantly increased ($p<0.0001$) serum tartrate-resistant acid phosphatase (TRAP) concentration (Table 11). Few studies have measured serum TRAP, however increases in the numbers of TRAP positive cells in vitro have been observed in models of rapid bone loss (Susi et al. 1966, Kalu 1990).

Vitamin E dose-dependently increased the serum antioxidant capacity. The ferric reducing ability (FRA) was significantly affected by diet (Table 11). A positive correlation ($r=.55$, $p<0.0001$) was established between final serum vitamin E and FRA. The FRA of rats consuming the 500 IU/kg vitamin E diet was 27.9% greater than the FRA of rats on the 15 IU/kg vitamin E diet. There appeared to be no effect of unloading on FRA. These results help to further solidify the effectiveness of vitamin E as an antioxidant.

Although vitamin E supplementation improved the antioxidant potential of the aged rats in this study, it did not provide protection against osteopenia. Twenty-eight days of hindlimb unloading induced significant osteopenia in the unloaded bones of aged rats. Along with bone loss, there were also alterations in the mineral components of

bone. The bone loss observed in this study was apparently a result of increased resorption and not diminished formation as reported in other studies. Further research into the etiology of age-associated bone loss occurring during unloading is needed due to the high incidence of osteoporosis and its related costs.

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Table 1. Vitamin E concentrations measured by high performance liquid chromatography (HPLC) on serum collected prior to hindlimb unloading and at necropsy from rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Baseline (μg/ml)	N	Final (μg/ml)
AMB, E-15	6	10.43±2.43	15	15.45±1.19
AMB, E-75	14	20.94±2.19	13	25.38±2.53
AMB, E-500	15	29.78±2.20	15	32.17±2.20
HLU, E-15	11	11.40±1.24	17	16.70±1.10
HLU, E-75	14	19.85±2.08	15	25.49±1.77
HLU, E-500	14	34.20±3.40	14	37.25±2.94
E-15	17	11.06±1.14^{c}	32	16.12±0.80^{c}
E-75	28	20.40±1.49^{b}	28	25.44±1.48^{b}
E-500	29	31.91±2.00^{a}	29	34.62±1.85^{a}
AMB	35	22.93±1.78	43	24.28±1.57
HLU	39	22.61±2.08	46	25.82±1.67
Source of Variation			*p*-values	
HLU		0.4850		0.1239
DIET		<0.0001		<0.0001
DIET*HLU		0.4734		0.3251

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 2. Final body weights of ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E diet.[1]

	N	Final Body Weight (g)	Change in Body Weight (g)
AMB, E-15	15	496±6	-4±7
AMB, E-75	15	499±8	5±7
AMB, E-500	16	494±9	11±7
HLU, E-15	16	463±8	-18±9
HLU, E-75	15	461±9	-16±11
HLU, E-500	14	472±12	-22±10
E-15	31	479±6	-8±6
E-75	30	480±7	-6±7
E-500	30	484±8	-5±7
AMB	46	497±5	7±4
HLU	45	465±5	-19±6
Source of Variation		*p*-value	
HLU		<0.0001	<0.0001
DIET		0.9400	0.9854
HLU *DIET		0.6700	0.5731

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 3. Corticosteriod levels measured in the serum and urine of ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Serum Corticosterone (ng/ml)	N	Urinary Corticosterone (ng/ml)
AMB, E-15	5	239.27±11.06	10	597.10±118.00
AMB, E-75	5	231.22±33.85	9	325.94±138.23
AMB, E-500	5	225.65±21.74	8	389.46±126.74
HLU, E-15	6	264.14±31.78	7	189.69±112.98
HLU, E-75	6	261.78±7.66	10	364.52±100.79
HLU, E-500	5	245.42±16.92	10	362.22±123.46
E-15	11	252.84±17.69	17	429.34±95.35
E-75	11	247.86±15.73	19	346.24±81.96
E-500	10	235.53±13.40	18	374.33±86.22
AMB	15	232.05±12.96	27	445.19±74.65
HLU	17	257.80±11.96	27	318.34±64.14
Source of Variation			*p*-values	
HLU		0.1930		0.1974
DIET		0.7787		0.9240
DIET*HLU		0.9731		0.1719

[1]Values represent means ± SEM.

Table 4. Humerus bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) measured by dual-energy x-ray absorptiometry (DEXA) of ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Humerus BMA (cm^2)	Humerus BMC (g)	Humerus BMD (g/cm^2)
AMB, E-15	15	1.43±0.015	0.317±0.005	0.221±0.002
AMB, E-75	15	1.46±0.018	0.327±0.007	0.223±0.002
AMB, E-500	16	1.43±0.020	0.314±0.007	0.220±0.002
HLU, E-15	16	1.43±0.014	0.308±0.004	0.215±0.002
HLU, E-75	16	1.41±0.016	0.299±0.005	0.212±0.002
HLU, E-500	15	1.39±0.016	0.300±0.006	0.215±0.002
E-15	31	1.43±0.010	0.312±0.003	0.218±0.001
E-75	31	1.44±0.013	0.312±0.005	0.217±0.002
E-500	31	1.41±0.013	0.307±0.005	0.217±0.002
AMB	46	1.44±0.010	0.319±0.004	0.221±0.001
HLU	47	1.41±0.009	0.302±0.003	0.214±0.001
Source of Variation			*p*-value	
HLU		<0.0188	<0.0002	<0.0001
DIET		0.1787	0.4964	0.9616
HLU*DIET		0.2821	0.2295	0.2218

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 5. Femur bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) measured by dual-energy x-ray absorptiometry (DEXA); femur dry weight and ash weight of ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Femur BMA (cm2)	Femur BMC (g)	Femur BMD (g/cm2)	Femur Dry Weight (g)	Femur Ash Weight (g)
AMB, E-15	15	2.5231±0.0309[a,b]	0.6594±0.0105	0.2613±0.0019	0.9609±0.0397	0.6902±0.0114
AMB, E-75	14	2.6015±0.0394[a]	0.6925±0.0140	0.2660±0.0018	1.0000±0.0686	0.7172±0.0269
AMB, E-500	16	2.5112±0.0355[a,b]	0.6490±0.0161	0.2579±0.0032	0.9302±0.0426	0.6711±0.0191
HLU, E-15	13	2.5123±0.0253[a,b]	0.6369±0.0089	0.2535±0.0025	0.8833±0.0317	0.6481±0.0114
HLU, E-75	15	2.4368±0.0278[b]	0.6139±0.0113	0.2518±0.0030	0.8657±0.0320	0.6336±0.0124
HLU, E-500	14	2.5041±0.0381[b]	0.6272±0.0169	0.2500±0.0035	0.8831±0.0440	0.6387±0.0169
E-15	29	2.5178±0.0198	0.6486±0.0071	0.2575±0.0017	0.9234±0.0262	0.6707±0.0090
E-75	29	2.5163±0.0281	0.6519±0.0115	0.2587±0.0022	0.9305±0.0385	0.6740±0.0162
E-500	30	2.5079±0.0255	0.6388±0.0116	0.2542±0.0025	0.9082±0.0304	0.6560±0.0130
AMB	45	2.5432±0.0208	0.6660±0.0083	0.2616±0.0015	0.9621±0.0290	0.6918±0.0117
HLU	42	2.4832±0.0181	0.6258±0.0073	0.2518±0.0017	0.8771±0.0204	0.6396±0.0204
Source of Variation				*p*-value		
HLU		<0.0254	<0.0003	<0.0001	<0.0002	<0.0004
DIET		0.9457	0.5309	0.1972	0.6600	0.4780
HLU*DIET		0.0346	0.0643	0.4431	0.4271	0.3021

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 6. Femur calcium, magnesium, copper, iron, and zinc concentrations determined by flame atomic absorption spectrophotometry in ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Calcium (mg/g)	Magnesium (mg/g)	Copper (μg/g)	Iron (μg/g)	Zinc (μg/g)
AMB, E-15	14	235.59±12.89	3.841±0.188	3.382±0.668	17.19±1.60	219.23±12.30
AMB, E-75	13	237.03±16.70	3.950±0.248	3.293±0.567	17.59±2.40	221.74±13.92
AMB, E-500	16	256.28±12.93	4.140±0.185	4.116±0.814	19.08±1.40	226.36±11.99
HLU, E-15	12	242.13±11.98	3.995±0.170	3.876±1.025	14.32±1.56	225.93±14.03
HLU, E-75	14	250.11±13.66	4.080±0.120	3.621±0.686	16.50±1.23	224.88±11.33
HLU, E-500	13	249.46±12.99	4.135±0.194	3.420±0.840	15.82±1.69	226.75±13.43
E-15	25	238.61±8.72	3.905±0.129	3.599±0.575	15.93±1.14	222.32±9.10
E-75	26	244.35±10.47	4.020±0.154	3.470±0.445	17.01±1.26	223.37±8.72
E-500	29	253.36±9.10	4.138±0.132	3.804±0.579	17.62±1.11	226.53±8.78
AMB	42	244.05±8.00	3.982±0.0117	3.636±0.410	18.03±1.00	222.61±7.15
HLU	48	247.38±7.34	4.076±0.1091	3.626±0.471	15.64±0.85	225.85±7.26
Source of Variation				*p*-value		
HLU		0.1354	0.1382	0.9130	<0.0086	0.0984
DIET		0.1536	0.1216	0.9171	0.2355	0.6814
HLU*DIET		0.4616	0.4487	0.6844	0.6195	0.4707

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 7. Femur total calcium, magnesium, copper, iron, and zinc content measured by flame atomic absorption spectrophotometry in ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Calcium (mg)	Magnesium (mg)	Copper (μg)	Iron (μg)	Zinc (μg)
AMB, E-15	14	221.52±6.73	3.624±0.107	3.20±0.60	15.95±1.16	205.74±5.90
AMB, E-75	12	226.62±10.82	3.780±0.153	3.10±0.52	15.83±1.33	212.17±8.06
AMB, E-500	16	233.90±10.20	3.792±0.143	3.75±0.75	17.19±0.96	205.56±7.62
HLU, E-15	11	214.71±8.04	3.609±0.124	3.51±1.01	12.54±1.24	199.48±8.03
HLU, E-75	14	210.19±6.24	3.436±0.089	2.98±0.48	13.93±0.95	189.54±4.03
HLU, E-500	13	212.58±5.35	3.489±0.086	2.87±0.66	12.97±1.03	189.82±4.55
E-15	25	218.38±5.12	3.617±0.079	3.33±0.55	14.45±0.90	202.85±4.82
E-75	26	217.42±6.00	3.594±0.090	3.04±0.34	14.81±0.81	200.40±4.89
E-500	29	224.76±6.49	3.652±0.090	3.35±0.51	15.30±0.80	198.50±4.83
AMB	42	227.72±5.37	3.731±0.077	3.38±0.37	16.39±0.64	207.51±4.12
HLU	38	213.37±3.73	3.501±0.056	3.09±0.40	13.20±0.60	192.77±3.27
Source of Variation				*p*-value		
HLU		<0.0393	<0.0291	0.6968	<0.0001	<0.0051
DIET		0.7975	0.9599	0.8753	0.4764	0.5349
HLU*DIET		0.5827	0.3597	0.6592	0.5307	0.3212

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 8. Third lumbar bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) measured by dual-energy x-ray absorptiometry (DEXA); third lumbar dry weight and ash weight; and density by displacement of ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet. [1]

	N	L3 BMA (cm^2)	L3 BMC (g)	L3 BMD (g/cm^2)	L3 Dry Wt (g)	L3 Ash Wt (g)
AMB, E-15	14	0.7654±0.0096	0.1879±0.0037^{b}	0.2454±0.0035	0.2697±0.0050	0.1728±0.0040
AMB, E-75	12	0.7854±0.0550	0.2156±0.0069^{a}	0.2565±0.0045	0.2990±0.0080	0.1963±0.0057
AMB, E-500	14	0.7837±0.0158	0.1946±0.0070^{b}	0.2473±0.0044	0.2837±0.0092	0.1785±0.0068
HLU, E-15	14	0.7684±0.0151	0.1734±0.0042^{c}	0.2256±0.0029	0.2550±0.0061	0.1583±0.0043
HLU, E-75	15	0.7599±0.0083	0.1737±0.0032^{c}	0.2284±0.0030	0.2551±0.0065	0.1593±0.0043
HLU, E-500	16	0.7679±0.0166	0.1726±0.0055^{c}	0.2243±0.0032	0.2548±0.0092	0.1602±0.0054
E-15	28	0.7669±0.0083	0.1806±0.0031^{b}	0.2355±0.0029	0.2623±0.0041	0.1655±0.0032^{b}
E-75	27	0.7712±0.0243	0.1923±0.0054^{a}	0.2409±0.0037	0.2746±0.0066	0.1757±0.0050^{a}
E-500	30	0.7753±0.0114	0.1829±0.0048^{b}	0.2350±0.0034	0.2683±0.0069	0.1687±0.0045^{a,b}
AMB	40	0.7778±0.0171	0.1985±0.0038	0.2494±0.0024	0.2834±0.0047	0.1818±0.0035
HLU	45	0.7654±0.0079	0.1732±0.0025	0.2261±0.0017	0.2550±0.0043	0.1593±0.0027
Source of Variation				*p*-values		
HLU		0.4111	<0.0001	<0.0001	<0.0001	<0.0001
DIET		0.8534	<0.0238	0.1096	0.1424	<0.0564
HLU*DIET		0.9211	0.0507	0.6221	0.2441	0.1051

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 9. Third lumbar calcium, magnesium, copper, iron, and zinc concentrations determined by flame atomic absorption spectrophotometry in ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Calcium (mg/g)	Magnesium (mg/g)	Copper (μg/g)	Iron (μg/g)	Zinc (μg/g)
AMB, E-15	14	199.31±2.70	3.61±0.071	4.69±0.118[a,b]	86.13±3.79	288.18±10.55
AMB, E-75	12	202.02±5.63	3.64±0.093	4.50±0.094[a,b]	113.39±14.35	282.67±5.37
AMB, E-500	14	198.29±2.05	3.59±0.074	4.38±0.106[b]	96.39±4.63	270.87±9.22
HLU, E-15	14	193.86±6.30	3.46±0.066	4.39±0.121[b]	92.22±7.71	264.03±10.03
HLU, E-75	15	189.33±3.08	3.58±0.115	4.69±0.141[a,b]	99.03±7.81	265.79±7.02
HLU, E-500	16	186.74±9.72	3.51±0.109	4.81±0.164[a]	83.71±4.31	267.14±6.45
E-15	28	196.58+3.41	3.54±0.0498	4.54±0.088	89.18±4.26[b]	276.10±7.51
E-75	27	194.70+3.16	3.61±0.0726	4.60±0.089	105.41±7.68[a]	273.29±4.78
E-500	30	192.32+5.15	3.54±0.0672	4.61±0.107	84.96±3.11[b]	268.88±5.42
AMB	40	199.71±1.96	3.61±0.045	4.52±0.064	94.40±5.06	280.47±5.17
HLU	45	189.89±3.94	3.52±0.058	4.69±0.086	94.46±3.89	265.72±4.42
Source of Variation						
HLU		<0.0382	0.2052	0.3031	0.5317	<0.0246
DIET		0.7758	0.6344	0.9245	<0.0172	0.4056
HLU*DIET		0.8122	0.9230	<0.0258	0.4420	0.3992

[1]Values represent means ± SEM.

Table 10. Third lumbar total calcium, magnesium, copper, iron, and zinc content measured by flame atomic absorption spectrophotometry in ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Calcium (mg)	Magnesium (mg)	Copper (μg)	Iron (μg)	Zinc (μg)
AMB, E-15	14	53.80±1.38	0.975±0.029	1.26±0.029	23.13±0.95	77.59±2.83
AMB, E-75	12	60.91±2.49	1.086±0.036	1.35±0.057	34.42±4.90	84.53±2.80
AMB, E-500	14	56.36±2.16	1.021±0.044	1.24±0.042	24.24±1.17	76.91±3.82
HLU, E-15	14	49.61±2.38	0.885±0.032	1.12±0.033	23.37±1.98	67.05±2.56
HLU, E-75	15	48.28±1.43	0.913±0.037	1.19±0.027	25.64±2.63	67.59±1.99
HLU, E-500	16	47.14±3.42	0.885±0.027	1.22±0.059	21.03±0.95	67.84±2.55
E-15	28	51.70±1.41	0.930±0.023	1.19±0.025	23.25±1.08[b]	72.32±2.13
E-75	27	53.62±1.81	0.990±0.030	1.26±0.033	29.55±2.71[a]	75.11±2.32
E-500	30	51.59±2.19	0.949±0.028	1.23±0.036	22.53±0.79[b]	72.08±2.36
AMB	40	56.73±1.21	1.025±0.022	1.28±0.025	26.91±1.71	79.43±1.89
HLU	45	48.31±1.44	0.894±0.018	1.18±0.025	23.30±1.14	67.51±1.35
Source of Variation				*p*-value		
HLU		<0.0001	<0.0001	<0.0025	<0.0335	<0.0001
DIET		0.4181	0.1170	0.2062	<0.0038	0.2863
HLU*DIET		0.2777	0.5520	0.1743	0.2025	0.2723

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 11. Serum parameters measured on blood collected at necropsy from ambulatory (AMB) and hindlimb unloaded (HLU) rats 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Alkaline Phosphatase (ukat/L)	Tartrate-Resistant Acid Phosphatase (u/L)	Ferric Reducing Ability of Plasma (μmol/L)	N	Osteocalcin (ng/ml)
AMB, E-15	15	1.65±0.13	4.43±0.29	553±35	10	23.65±3.64
AMB, E-75	15	1.57±0.08	4.27±0.24	575±20	11	24.13±3.58
AMB, E-500	16	1.85±0.17	4.04±0.21	659±35	12	26.45±3.92
HLU, E-15	17	1.60±0.12	5.73±0.17	533±26	12	27.97±3.17
HLU, E-75	16	1.54±0.13	5.47±0.23	578±20	12	27.21±3.55
HLU, E-500	16	1.82±0.14	5.38±0.23	727±47	12	27.58±3.28[2]
E-15	32	1.62±0.09	5.12±0.20	542±21[b]	22	26.01±2.38
E-75	31	1.56±0.08	4.91±0.20	576±14[b]	23	25.74±2.49
E-500	32	1.83±0.11	4.71±0.19	693±30[a]	24	26.99±2.52
AMB	46	1.69±0.08	4.24±0.14	597±19	33	24.83±2.10
HLU	49	1.65±0.08	5.53±0.12	611±22	36	27.59±1.88
Source of Variation			*p*-values			
HLU		0.7265	<0.0001	0.5004		0.1718
DIET		0.0990	0.2346	<0.0001		0.7959
HLU*DIET		0.9911	0.9688	0.3544		0.8764

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).
[2]One animal from this group was omitted due to an unusually high value.

Table 12. Alkaline phosphatase (ALP) and total protein extracted from the femur of ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Bone ALP (ukat/mg soluble bone protein)	Bone Protein (mg protein/whole bone)	Bone Protein (mg protein/g bone)
AMB, E-15	14	17.64±3.86	0.809±0.106	0.865±0.126
AMB, E-75	15	17.50±7.04	1.110±0.150	1.230±0.178
AMB, E-500	16	16.80±3.40	0.955±0.117	1.037±0.131
HLU, E-15	16	19.68±3.34	0.663±0.104	0.763±0.119
HLU, E-75	16	20.54±3.63	0.689±0.101	0.836±0.133
HLU, E-500	16	17.43±4.68	0.824±0.074	0.995±0.110
E-15	30	18.73±2.50	0.731±0.074	0.810±0.086
E-75	31	19.07±3.83	0.892±0.096	1.027±0.114
E-500	32	17.11±2.84	0.889±0.069	1.016±0.084
AMB	45	17.29±2.83	0.961±0.073	1.048±0.086
HLU	48	19.22±2.23	0.725±0.054	0.865±0.070
Source of Variation		*p*-value		
HLU		0.6462	<0.0103	0.0762
DIET		0.8790	0.2887	0.1893
HLU*DIET		0.9722	0.3111	0.2599

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

CHAPTER V

CELL CULTURE

RESULTS AND DISCUSSION

The primary goal of bone research is an understanding of bone cell biology in the living organism. Bone cell culture has developed as a means of isolating bone cells to produce cell populations of known composition that employ the characteristics of cells in vivo and allow researchers to study the systemic and local factors that affect bone, in an in vitro environment. Thus, "any understanding of bone cell regulation is a key ingredient in understanding not only the development, maintenance, and repair of the skeleton, but also the prevention and treatment of skeletal disorders" (Marks & Popoff 1988).

The impact of hindlimb unloading and vitamin E status on cultures of marrow-derived bone cells was examined as part of this study. Throughout the course of this study many problems arose which affect the results of this chapter. Primary bone cell culture is a very delicate science and generally requires much practice and experience to perfect the technique. The large number of animals in this study and the three separate replications of the experiment provided an excellent opportunity to develop cell culture technique. Cells obtained from animals in replication #1 of the experiment were used to establish optimal assay conditions. These cells were cultured in different media and with

varying growth factors to determine the most appropriate methodology for the rest of the experiment. We planned to use cells obtained from the animals of replications #2 and #3 to provide the actual data and results for the experiment. However, during the incubation period of replication #2 cell death occurred prior to termination of the experiment. This dramatically reduced the number of samples in the experiment and increased dependence on replication #3 to provide results. Cells from replication #3 were successfully cultured until pre-confluency and harvested for measurement of bone remodelling markers. Photographs were taken throughout the course of cellular differentiation and growth, and are included in Appendix B. However, attempted measures of cellular secretion of alkaline phosphatase (ALP), osteocalcin, and tartrate-resistant acid phosphatase (TRAP) were unsuccessful.

Alkaline phosphatase, a marker of bone formation, was measured on the lysate of sonicated cells and in the cell media supernate. No detectable levels of ALP were observed in the lysate and minute levels were measured in the supernate. These results could possibly be attributed to the sensitivity of the kit used to measure ALP. The lysate and supernate ALP were measured with a kit that is normally used to detect ALP levels in plasma and serum. A more sensitive procedure might have produced valid results. In a similar study Machwate et al. (1993) measured the ALP activity in bone marrow cells obtained from rats subjected to 14 days of hindlimb unloading. Their technique involved scraping the cell layer into distilled water and then sonicating the cells. In this study, cells were sonicated while still attached to the wells. Sonicating cells while in the well was very awkward and difficult and could possibly be a reason that no detectable levels of ALP were detected in the lysate. In the future, using a more sensitive measure for

detecting ALP and detaching cells from the well prior to sonicating may help to eliminate some of this error.

Osteocalcin, also a marker of bone formation was measured in the cell media supernate by radioimmunoassay. Again, no detectable levels of osteocalcin were observed in the supernate. Although the kit was appropriate for measuring osteocalcin in cell culture media, the osteocalcin concentration in the supernate was apparently below the minimum threshold detectable by the kit.

To measure bone resorption, cell plates were stained for TRAP positive cellular activity. Osteoclasts, bone resorbing cells, secrete the acid phosphatase enzyme to break down bone mineral and bone matrix. Tartrate-resistant acid phosphatase staining was performed using a commercially available kit (Sigma). However, in our experiment, tartrate-resistant cells were not distinguishable from other cells. Lack of experience in cell staining is the most likely cause of this error. Others staining for TRAP activity have reported increases in the number of TRAP-positive cells in cultures obtained from models undergoing rapid bone loss (Kalu 1990).

All cells were plated in 24-well plates at a similar initial density. To accomplish this, initial bone cell viability was measured in the bone marrow of each rat. Data is reported from replications #2 and #3 of the experiment (Table 1). Hindlimb unloading significantly reduced the initial number of viable bone marrow cells. A diet effect ($p<0.0471$) was also observed in initial cell viability. Animals on the 75 IU/kg vitamin E diet had significantly more viable cells than animals on the 500 IU/kg diet. However, cell viability did not differ significantly between animals on the 15 IU/kg and 75 IU/kg diets, or between animals on the 15 IU/kg and 500 IU/kg diets. This indicates that

vitamin E at the 75 IU/kg diet was more effective than the 500 IU/kg diet at protecting the cellular integrity of marrow cells.

In addition to initial cell viability, percent of viable versus non-viable cells was assessed. However, there was no significant affect of HLU or diet on the percentage of viable cells (Table 1).

The main goal of bone cell culture is to isolate cell populations of known composition. However, none of the common methods currently used to isolate bone cells produce a pure population, where every cell type is identifiable. Because osteoclasts are derived from hemopoietic origin, bone marrow cultures have been used primarily to study their biology. Osteoblasts, however, have been isolated primarily by the sequential enzymatic digestion of bones. The failure of this experiment to observe biochemical indicators of bone formation in marrow cultures may be attributed to the fact that osteoclastic activity in the cultures was far outweighing osteoblastic activity in the cultures, regardless of diet or HLU. However, because no data are available to determine osteoclastic activity, this cannot be determined. Additionally, altered bone remodeling displayed in vitro may be a result of culture conditions and not necessarily the physiological state of the animal. Although bone cell culture provides valuable insight into bone physiology, its limitations must be considered when extrapolating the information to bone remodeling in vivo.

Table 1. Initial cell viability and percent of viable bone marrow cells obtained from ambulatory (AMB) and hindlimb unloaded (HLU) rats fed the 15, 75, or 500 IU vitamin E/kg diet.[1]

	N	Initial Cell Viability (Total Viable Cells)	Percent Cell Viability (% Viable Cells)
AMB, E-15	10	$6.61 \times 10^6 \pm 5.21 \times 10^5$	93.53±0.69
AMB, E-75	9	$7.39 \times 10^6 \pm 4.63 \times 10^5$	94.63±0.53
AMB, E-500	12	$5.95 \times 10^6 \pm 3.73 \times 10^5$	94.45±0.78
HLU, E-15	11	$5.44 \times 10^6 \pm 3.17 \times 105$	94.79±0.54
HLU, E-75	11	$5.76 \times 10^6 \pm 3.02 \times 10^5$	94.22±0.66
HLU, E-500	12	$5.15 \times 10^6 \pm 4.32 \times 10^5$	93.04±0.46
E-15	21	$6.00 \times 10^6 \pm 3.19 \times 10^{5a,b}$	94.16±0.45
E-75	20	$6.49 \times 10^6 \pm 3.19 \times 10^{5a}$	94.40±0.42
E-500	24	$5.55 \times 10^6 \pm 2.91 \times 10^{5b}$	93.77±0.48
AMB	31	$6.58 \times 10^6 \pm 2.72 \times 10^5$	94.20±0.40
HLU	34	$5.44 \times 10^6 \pm 2.07 \times 10^5$	94.02±0.34

Source of Variation	*p* value	
HLU	<0.0006	0.7408
DIET	<0.0471	0.5200
DIET*HLU	0.6076	0.0756

[1]Values represent mean ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

Summary

The effects of hindlimb unloading, vitamin E, and their interaction on bone were investigated. Using a 2 X 3 factorial design, ninety-seven, 11-month old, male, Sprague Dawley rats were either hindlimb unloaded (HLU) or remained ambulatory (AMB) as controls. After 10 days on a diet containing no vitamin E, rats in each group were randomly assigned to one of three dietary treatments containing 15, 75, or 500 IU *dl*-α-tocopherol/kg diet, which was consumed for the 2 months prior to unloading. After 28-days of HLU, rats were necropsied and bones were collected for determination of bone mineral density and mineral concentrations. Selected biochemical measures of bone formation and resorption were also performed. Serum vitamin E was dose-dependently elevated ($p<0.0001$) by dietary vitamin E treatment. Bone resorption, as indicated by serum tartrate-resistant acid phosphatase, was elevated ($p<0.0001$) in unloaded animals, but was not affected by diet. Serum and bone alkaline phosphatase and serum osteocalcin, markers of bone formation, were not significantly affected by diet or unloading. Hindlimb unloading significantly reduced bone protein extracted from the femur. Bone mineral density, measured by dual-energy x-ray absorptiometry (DEXA), of the third lumbar vertebrae (L3), femur, and humerus was significantly reduced by HLU,

but not by diet. HLU significantly reduced iron concentration in the femur of unloaded animals; furthermore, HLU significantly decreased the total content of calcium, magnesium, iron, and zinc in the femur. In L3, HLU significantly reduced the total content of calcium, magnesium, copper, iron, and zinc. Diet had no effect on mineral in the femur or L3, except that, the L3 of animals on the 75 IU diet had higher total ($p<0.0172$) bone iron when compared to animals on the 15 IU and 500 IU diets. Hindlimb unloading significantly reduced the total number of viable bone marrow cells. Vitamin E at 75 IU/kg diet increased ($p<0.0471$) cell viability when compared to the 500 IU/kg diet.

Results of Hypothesis Testing

The following hypotheses were developed for this study:

1. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter the bone mineral area (BMA), bone mineral concentration (BMC), or bone mineral density (BMD) in aged rats.

Hypothesis #1was rejected because DEXA scans showed significant reductions in BMD of the humerus, L3, and femur of hindlimb unloaded animals. A diet and HLU interaction was observed in L3. Third lumbar BMC was significantly less in hindlimb unloaded animals compared to ambulatory, regardless of diet. Ambulatory animals on the 75 IU/kg diet had significantly greater BMC of the L3 compared to ambulatory animals on the 15 and 500 IU/kg diets. An interaction between diet and HLU was observed in femur BMA. Femur BMA in ambulatory animals on the 75 IU/kg diet was

significantly greater than femur BMA of hindlimb unloaded animals on the 15 and 500 IU/kg diets.

2. There will be no statistically significant effects of hindlimb unloading, vitamin E status, or their interaction on biochemical markers of bone remodeling (i.e. serum alkaline phosphatase, bone-specific alkaline phosphatase, serum osteocalcin, and serum tartrate-resistant acid phosphatase) in aged rats.

Hypothesis #2 was partially rejected because HLU increased ($p<0.0001$) the serum TRAP concentration. However, no significant diet or HLU effect was observed in serum ALP, bone-specific ALP, or serum osteocalcin.

3. There will be no statistically significant difference in the total bone content or concentration of bone calcium, magnesium, copper, iron, and zinc due to hindlimb unloading, vitamin E status, or their interaction in hindlimb unloaded rats.

Hypothesis #3 was rejected because HLU reduced ($p<0.0086$) iron concentration in femur. Hindlimb unloading also significantly decreased the total content of calcium, magnesium, iron, and zinc in the femur. In L3, HLU significantly reduced the total content of calcium, magnesium, copper, iron, and zinc. Diet had no effect on mineral in the femur or L3, except that, the L3 of animals on the 75 IU vitamin E/kg diet had higher ($p<0.0172$) total bone iron when compared to animals on the 15 and 500 IU/kg diets.

4. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter the initial viability, alkaline phosphatase secretion, osteocalcin secretion, or tartrate-resistant acid phosphatase activity in marrow-derived bone cells from aged rats.

Hypothesis #4 was partially rejected because HLU significantly reduced the initial viability of marrow-derived bone cells. The 75 IU/kg vitamin E diet increased

($p<0.0471$) initial cell viability when compared to the 500 IU/kg diet. However, no other reliable cell culture data are available for hypothesis testing.

Conclusions and Recommendations

Although vitamin E supplementation improved the antioxidant potential of the aged rats in this study, it did not provide protection against osteopenia. Twenty-eight days of hindlimb unloading induced significant osteopenia in the unloaded bones of aged rats. Along with bone loss, there were also alterations in the mineral components of bone. The bone loss observed in this study was apparently a result of increased resorption and not diminished formation as reported in other studies. Further research into the etiology of age-associated bone loss occuring during unloading is needed to better clarify the results from this study.

With the exception of initial cell viability, no reliable data were acquired for exploring the impact of hindlimb suspension and vitamin E status on marrow-derived bone cells. However, what little data were available provided valuable information that vitamin E in a "moderate" dose was most effective at protecting cellular integrity in marrow cells, and that at "high" doses vitamin E may be detrimental, rather than beneficial.

A similar vitamin E effect was observed on other parameters of bone composition. It appeared that vitamin E was most protective in a "moderate" dose. Although assumptions can be made from these results, more in-depth research studies must be performed before valid conclusions can be made regarding vitamin E and its effect on bone.

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APPENDICES

APPENDIX A

OKLAHOMA STATE UNIVERSITY

OSU

College of Veterinary Medicine
Laboratory Animal Resources Unit
Stillwater, Oklahoma 74078-2002
405-744-7631

May 27, 1998

Institutional Animal care and Use Committee Action

This protocol was reviewed by the IACUC with the following action:

Protocol Title: "Effects of iron Overload on Bone Formation and Bone Resorption in Tail Suspended Rats"

Protocol Number: 733

Principle Investigator: Dr. Barbara Stoecker

Animal Number and Species: 48 SD Rats

Expiration Date: May 31, 2001

Approval ✓ Deferral _____

Approval with Modifications _____

Comments:

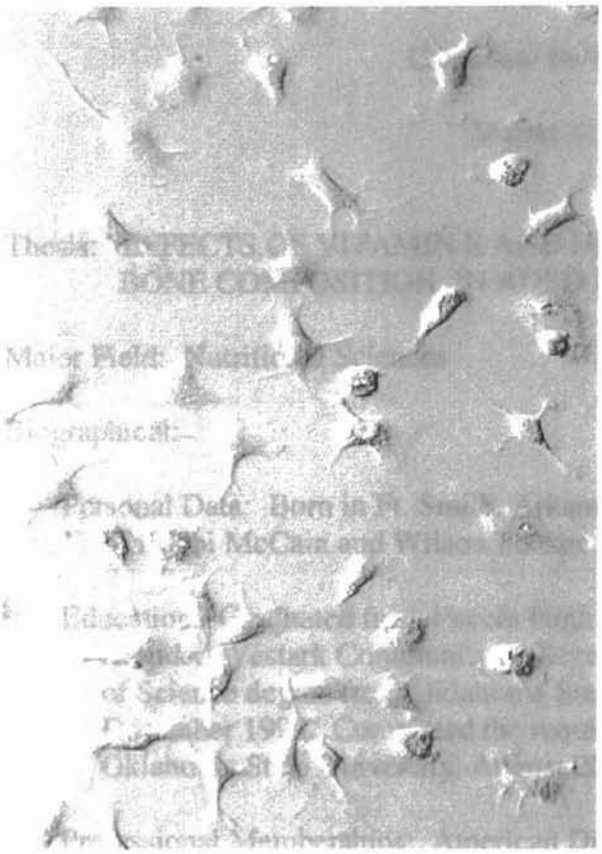
Date of final Institutional Committee Action: May 21, 1998

Signature of IACUC Chairman _____ Date 6/1/98

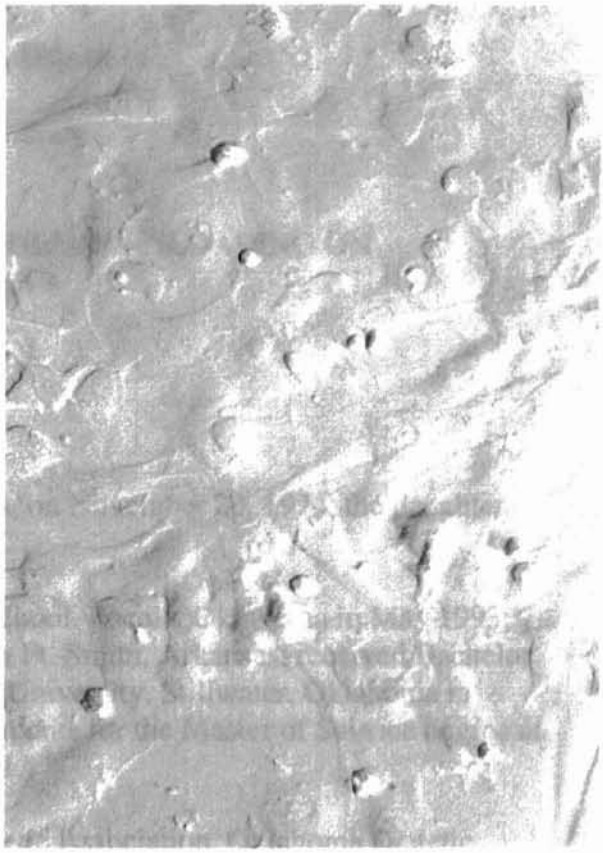
Signature of IACUC Veterinarian _____ Date 5/27/98

Institutional Assurance Number A3722-01

APPENDIX B



1. Bone marrow cells a few days into culture.



2. Bone marrow cells reaching pre-confluency near 2 weeks of culture.

VITA 2

Tracy Lynn Riggs

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF VITAMIN E AND HINDLIMB UNLOADING ON BONE COMPOSITION IN AGED RATS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Ft. Smith, Arkansas, on November 20, 1974, the daughter of Bobbi McCain and Wilson Tucker III.

Education: Graduated from Pocola High School, Pocola, Oklahoma in May 1993; attended Westark Community College in Ft. Smith, Arkansas; received Bachelor of Science degree from Oklahoma State University, Stillwater, Oklahoma in December 1998. Completed the requirements for the Master of Science degree at Oklahoma State University, August 2001.

Professional Memberships: American Dietetic Association, Oklahoma Dietetic Association